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Icariin stimulates myeloid Leukemic cells differentiation by increasing smad4 protein abundance leading to the inhibition of myeloid Leukemic cell growth

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Abstract. As acute myeloid leukaemia (AML) is still a highly frequent disease (4.3 per 100,000 patients) worldwide and produces an incredibly high incidence rate (4 per 100,000), finding a more effective treatment or new direction of treatment is essential. This research aims to find a new direction of treatment by investigating cell differentiation. Focusing on the BMP signaling pathway, especially the protein of Smad4, the potential of increasing the chance of cell differentiation was found. Icariin (ICAR) was used to target the Smad4 pathway and hopefully become a potential treatment. Therefore, the hypothesis is set as the ICAR would increase the Smad4 level, which activates cell differentiation and potentially become a new treatment for AML. The experimental proposal was concluded in this research, and the combination of imaginary results was analyzed.

Keywords: AML, BMP Signaling Pathway, Differentiation.

1. Introduction

1.1. Epidemiology and Etiology of AML

Like other cancers, AML is also caused by genetic reasons and leads to uncontrollable cell proliferation. Leukaemia is a relatively common cancer, with 4.3 per 100,000 patients in the USA and especially in the old age group (27.4 per 100,000 patients for the 70-year group) [1]. This type of cancer is widely spread and has a high incidence rate (4 per 100,000) [2]. The comparison of the probability of getting the disease and the incidence rate suggests that leukaemia leads to an incredibly high death rate. The average 2-year survival probability for patients with myeloid leukaemia is only 16.3% [3].

1.2. Current Treatments

The treatments of acute myeloid leukaemia have been investigated for several decades. These treatments are mainly classified into intensive and non-intensive methods [4]. Most of the chemotherapies belong to intensive treatments, including the FLT3 inhibitors [5], CD33-targeting drugs, and other standard oncology targets (p53 and cyclin-dependent kinase inhibitors) [6]. The non-intensive methods were used primarily for aging patients because curing leukaemia in elderly patients sometimes cannot be possible

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and induces lots of pain. These treatments include LDAC and HMA [7]. However, the high death rate suggested that the treatments for leukaemia required further research.

1.3. New Discussed Hallmark of Cancer

Cell differentiation ability has been discovered as a new hallmark of cancers [8]. The hallmark of cancer was investigated for researchers to understand the cancer genesis and the difference between cancer cells and healthy cells. The point mentioned in this paper is that cancer cells usually lose their ability to differentiate and undertake clonal proliferation. Increasing the differentiation ability of cancer cells may potentially reduce the speed of cancer cell proliferation. Theoretically, this would be a new potential for cancer treatment. Among them, Smad4 is a typical example of a protein that leads cancer cells to undergo differentiation. Smad4 is a transcription factor (TF) involved in the BMP signaling pathway [8].

1.4. Relationship between Smad4 and AML

In some instances of AML, the cancer cells are caused by mutations in the Smad4 gene [9]. The failure of the Smad4 gene would reduce its proteins' activity, reducing the chance of DNA binding [10]. Smad4 is a protein the serine-threonine receptor activates and becomes phosphorylated during activation [11]. Smad4 can regulate cell differentiation by inhibiting the receptors like TGF-beta, Activin, and Nodal receptors [12]; however, whether the upregulation or the downregulation of differentiation may also depend on other signaling molecules present in the cells, such as Taz and RUNX2 [13]. Therefore, previous research suggested that the loss or mutation of Smad4 genes did induce cancer, and there is a relationship between the Smad4 receptor and cell differentiation.

1.5. Introduction of Icariin

Icariin (ICAR) is a compound extracted from the Chinese traditional medicine – Epimedium. In Chinese traditional treatment, it is used to increase the cardiac output, the ability of angiogenesis, and strengthen the immune system and bone metabolism. Occasionally, it is also a drug used to treat kidney deficiency [14]. A critical property of ICAR concentrated in this research is that it can potentially increase the chance of cell differentiation via the BMP/Smad4 signaling pathway [15, 16]. However, the evidence above suggests that ICAR could possibly be a treatment in oncology. The application of ICAR on AML has not been clinically used. In this research, we will focus on whether ICAR increases the level of Smad4, whether the increased level of Smad4 leads to cell differentiation, and whether cell differentiation eliminates ML cell growth.

1.6. Hypothesis

The hypothesis I predicted, according to the background information, is: Increasing the concentrations and treatment durations of Icariin increases the chance of myeloid leukemic (ML) cell differentiation by raising the Smad4 level and leads to inhibiting ML cell growth. Several experimental techniques will test this hypothesis in this research. The presence of CD157 is measured with FACS techniques to monitor ML cell differentiation. The level of Smad4 is measured by western blot, and MTT measures the growth of cancer cells.

2. Methods

2.1. Reagents

Pure ICAR is purchased from drug companies, and the stock solution of ICAR is prepared with methanol (1mg/ml). The effective dose of ICAR investigated by researchers was between 0.01 to 1 μg/ml [17]. Therefore, the stock solution is diluted into the listed concentrations (1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 ng/ml). All solutions should be kept at -20°C.

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2.2. Cell lines

To investigate the cell differentiation purpose, a cell line for the M0 type of AML (type of AML with minimal differentiation) is used, the Kasumi-3 cell line [18]. The myeloid cells extracted from a healthy volunteer are used as the control (CCL). Both cell lines are purchased from ATCC companies. Those cells grow at 37 °C, supplying 5 % CO¬2, 18.6% O2, and at least 95% humidity. The pH should be kept at around 7.4. They grow in serum, providing the essential hormones and nutrition for cell line growth.

2.3. Western blot

Both control (CCL) and sample cell line (Kasumi-3) were treated with different concentrations (listed in reagent sections and the positive control 100nM 5-FU) and different time lengths after receiving the drugs (0h, 3h, 5h, 10h, 24h, 48h) are added in the RIPA buffer. They are mixed in Roche solution at 2℃ for 10 mins, then heated for 5 min at 95℃. Those samples are then added to the well of SDS – polyacrylamide (SDS page) gels. The negative control used with the solution was mentioned only without any cells. After separation, protein segments are transferred onto the immobilon-PVDF transfer membrane. After waiting for 12h, TBST is used to wash this membrane three times. The appropriate secondary antibodies were added onto the membrane for 1 hour at room temperature. SuperSignal West Dura Extended Duration Substrate scans the transfer membrane, and the protein bands become visible.

2.4. FACS

Cell differentiation is measured with the presence of antigen CD157. Like the Western blot, both control (CCL) and sample (Kasumi-3) cell lines are used to investigate whether there is an increase in the differentiation. But the positive control for this experiment has not been investigated. So, there is no positive control. The known cell number concentration cells are washed (single cell suspension) and set in an ice-cold FACS buffer. As same as the experiment introduced before, the negative control would use with the buffer only. The antigen CD157 present on the surface of the cells is labeled with cold fluorescence labeling. Cells were then plated onto the 6-well plates and analyzed by a BD FACS Aria III flow cytometer. Then, the data is further analyzed by the software (FlowJo 7.6 software).

2.5. MTT

CCL, Kasumi cells (1000 cells/well) are placed in the 96-well plates for 24h. Then, cells are added with different concentrations of ICAR and the positive control – Taxol 100nM. All cells are treated with the same volume of the culture median. Negative control was used with just the culture median. After this pre-treatment, a 0.02ml MTT solution is supplemented into each well for 4 hours. Then, add another 0.15ml dimethyl sulfoxide to dissolve the crystals. The results are read by the microplate reader (Bio-Rad).

2.6. Statistical analysis

For the experiments listed above, the appropriate statistical analysis should be carried out to measure whether the effects produced by ICAR are different on cancer cells and healthy cells. The paired – T test is used to suggest where there is a significant difference between the CCL and the Kasumi-3 at a given concentration. The relationship between the concentration of ICAR and the responses was analyzed by constructing the dose-response curve and comparing their parameters. If the dose-response curve is unable to be drawn or the R squared value is too small (<0.5), then it suggests that ICAR did not produce the response investigated. Every experiment was repeated until we saw three concordant results. The similarity of those results was tested also by paired - T test to test whether there was a statistical difference between those results. For concordant results, the difference should be minimal (i.e., no statistical differences).

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3. Results

3.1. Possible results

Due to the time and lab availability, the experiment has not been carried out yet. The combination of imaginary results has been tabulated in Table 1.

Table 1. A combination of possible results

Combination of possible results (CR)	Smad4 proteins increase in Kasumi-3 by Western blot compared with CCL?	CD157 level increases in Kasumi-3 by FACS compared with CCL?	ML cell growth decreases in Kasumi-3 by MTT compared with CCL?	Hypothesis is …
CR1	+	+	+	Fully supported
CR2	+	+	-	Partially supported
CR3	+	-	+	Partially supported
CR4	+	-	-	Partially supported
CR5	-	+	+	Partially supported
CR6	-	+	-	Partially supported
CR7	-	-	+	Partially supported
CR8	-	-	-	Fully contradicted

Note: Table 1. Combination results. + indicates the measurements go with the same direction indicated in the table - indicates the measurements do not go with the direction indicated in the table.

3.2. Explanation of the result table

CR1: The Smad4 level of Kasumi-3 (M0 cell line) measured by Western blot increases with ICAR concentration, and there should not be any increase in the CCL (control cell line). As time increases, the Smad4 level should increase, followed by a decrease. The CD157 level also increases with ICAR concentration. The cell growth decreases with an increasing ICAR concentration. The hypothesis is fully accepted with those results.

CR2: The Smad4 level of Kasumi-3 (M0 cell line) measured by Western blot increases with ICAR concentration, and there should not be any increase in the CCL (control cell line). As time increases, the Smad4 level should increase, followed by a decrease. The CD157 level also increases with ICAR concentration. The cell growth increases or does not change with an increasing ICAR concentration. The hypothesis is only partially accepted with these results.

CR3: The Smad4 level of Kasumi-3 (M0 cell line) measured by Western blot increases with ICAR concentration, and there should not be any increase in the CCL (control cell line). As time increases, the Smad4 level should increase, followed by a decrease. The cell growth still decreases with an increasing ICAR concentration. However, the CD157 level decreases or does not change with increasing ICAR concentration. The hypothesis is only partially accepted with these results.

CR4: The Smad4 level of Kasumi-3 (M0 cell line) measured by western blot increases with ICAR concentration, and there should not be any increase in the CCL (control cell line). As time increases, the

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Smad4 level should increase, followed by a decrease. The CD157 level decreases or does not change with increasing ICAR concentration. The cell growth increases or not change with an increasing ICAR concentration. The hypothesis is only partially accepted with these results.

CR5: The Smad4 level of Kasumi-3 (M0 cell line) measured by western blot decreases or does not change with ICAR concentration, and there should not be any increase in the CCL (control cell line). However, the CD157 level still increases with ICAR concentration, and cell growth decreases with an increasing ICAR concentration. The hypothesis is only partially accepted with these results.

CR6: The Smad4 level of Kasumi-3 (M0 cell line) measured by western blot decreases or does not change with ICAR concentration, and there should not be any increase in the CCL (control cell line). The cell growth increases or does not change with an increasing ICAR concentration. But the CD157 level increases with ICAR concentration. The hypothesis is only partially accepted with these results.

CR7: The Smad4 level of Kasumi-3 (M0 cell line) measured by western blot decreases or does not change with ICAR concentration, and there should not be any increase in the CCL (control cell line). The CD157 level decreases or does not change with increasing ICAR concentration. The cell growth decreases with an increasing ICAR concentration. The hypothesis is only partially accepted with these results.

CR8: The Smad4 level of Kasumi-3 (M0 cell line) measured by western blot decreases or does not change with ICAR concentration, and there should not be any increase in the CCL (control cell line). The CD157 level decreases or does not change with increasing ICAR concentration. The cell growth increases or does not change with an increasing ICAR concentration. The hypothesis is entirely contradicted by these results.

4. Discussion

4.1. Discussion on the ICAR effects on the CCL

Using a drug is inappropriate if it responds to normal healthy cells at its effective concentration. So, the hypothesis would be rejected if the drug's effect on Kasumi-3 is not significantly higher than the healthy cells (CCL). So, in this discussion, it was assumed that ICAR has no effect on control in any CRs.

4.2. Discussion on CRs

For the first four results (CR1 – CR4), the level of Smad4 increases only on the Kasumi-3 cell lines. This means that the ICAR increases the Smad4 only in ML cells rather than the normal healthy cells. So, the first part of the hypothesis is supported: ICAR increases the Smad4 protein levels.

CD157 is an antigen only found when the cells undertake the differentiation process. The level of CD157 suggested the chance of cells undertaking the differentiation process. As the CD157 levels increase, the differentiation probability increases. However, in the first four cells discussed (CD1-CD4), only results (CR1, CR2) suggested the increases in CD157 level. This can be explained by the introduction that Smad4 is a vital protein required in the BMP signaling pathway, stimulating cell differentiation. CR1 and CR2 support this hypothesis: the increasing Smad4 levels increase the chance of differentiation.

For the CR1, the ML cell growth decreases, suggesting that ICAR inhibits the ML cell growth. The hypothesis is fully supported with CR1. Nevertheless, a vast process is still required for ICAR to become an oncological treatment. For example, drug metabolism, side effects, effective dosing, toxic dosing, and other metabolic reaction should be investigated before bringing the drug to the later stage of clinical trials. To explore those parameters, the drug should be tested in vivo. For example, drug metabolism is measured by changes in the concentration of blood in mice or even the changing drug concentration in the area. The data collected from mice is different from the data collected from the human. So, probably the dosage in the body should be carefully tested before giving it to the patients.

There are no noticeable CD157 level increases in the CR3 and CR4 results suggest that although the level of Smad4 increases, the probability of cell differentiation does not increase. If three concordant results are recorded, and there are no operational issues, this might be explained by other cell molecules

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inhibiting cell differentiation. As the introduction suggests, cell differentiation depends on other signaling molecules, such as Taz and RUNX2. Only changing one protein level is not satisfied with increasing cell differentiation. Therefore, the combination of drugs might be used in terms of increasing the cell differentiation probability.

Even if the level of differentiation increases, the results (CR2 and CR4) suggest that the effectiveness of differentiation in eliminating the ML cells' growth is still minimal. The reason might be that only activating certain cell differentiations may not be sufficient to reduce tumor growth. Also, activating the BMP signaling pathway by increasing the Smad4 level may enhance cell dedifferentiation. This is the process to generate the cancer stem cell; therefore, increasing the Smad4 level may also increase the possibility of cancerogenesis. On the other hand, it is still an assumption that activating cell differentiation will control tumor growth. No direct evidence supports this assumption, especially on AML cancers (a highly complex cancer in terms of treatment). Although the differentiation level increases, tumor growth may not be controlled.

Interestingly, CR3 suggested a Smad4 level increase and, as the prediction, the ML cell growth decreased. Nevertheless, the level of CD157 is not increased. This suggested that the ML cell growth is controlled by the Smad4 protein level, however, not by increasing the differentiation level. The BMP signaling pathway is not only involved in the cell differentiation process but also involved in the cell proliferation process. Maybe some mechanism there would induce to decrease in cancer cell growth.

For the last four results (CR5 - CR8), there is no directional linkage between the ICAR concentration and the Smad4 levels. This suggested that the ICAR did not increase the Smad4 levels in cancer cells. Although previous evidence suggests this is unlikely to happen, cancer cells, especially the AML cells, are complex. The ICAR may only work on certain tissue types rather than ML cells. Alternatively, the place of metabolism of ICAR is particular (only metabolized in the bone), so they cannot target the tumor cells in the blood. This is unlikely to happen, as the information seen in the ICAR introduction. Further investigation on the properties of ICAR should be carried out to understand its mechanism.

Results (CR5 and CR6) suggest no relationship between the Smad4 levels and the cell differentiation level. Even if the Smad4 level decreases, the probability of cell differentiation increases. This suggests that the ICAR works on increasing the cell differentiation level by other mechanisms. This is possible as the ICAR has massive functions in Chinese medicine clinics. It may increase the cell differentiation level by other signaling pathways or simply switch on specific genes. CR5 shows the assumed result that as the probability of cell differentiation increases, the cancer cells reduce growth. However, CR6 suggests that even though the cell differentiation chance increases, the cancer cells are still growing. The explanation for that is similar to CR2: it did not support the hypothesis that the cancer cells decrease where the cell differentiation probability increases.

Result CR7 suggested that even though there is no increase in Smad4 and CD157 levels, there is a potential to inhibit tumor growth. This means the ICAR works through other pathways to kill cancer cells. Although the pathway is not as the research predicted, this drug may still be used to treat AML cells. Then, further research is needed to investigate the pathway of this drug. For example, monitoring the cell apoptosis rate, cell proliferation rate and even the function of other healthy cells involved in the immune system (B and T cells). As the mechanism of ICAR is precise, this drug may still be taken through clinical trials.

Result CR8 is fully contradicted by the hypothesis, as there is no potential use for ICAR to treat AML. This is caused by the ED50 working on another disease that might differ from the AML treatment. So, positive results may be shown by increasing the concentration of ICAR. The standard metabolism time length of ICAR is within the first 10 hours. The time duration for measuring the Smad4 levels is up to 48 hours. A negative result suggested that ICAR did not increase the level of Smad4. However, for the MTT test, no time duration is measured, so the negative result may be that there is insufficient time for the drug to interact. This experiment might be repeated by increasing the reaction time.

The results (CR3, CR5, and CR7) all suggested a potential for decreasing cancer cell growth. Although the pathway is not like what has been predicted, it suggests that ICAR may still be a therapeutic measure for AML. However, the pathway should be clarified. For CR3, the effects of BMP signaling

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pathway on cell proliferation might be tested. Instead of measuring the level of CD157, AURKB protein can be measured by western blot. CR5 suggests that increasing cell differentiation probability reduces the cancer cells so that ICAR may work on another pathway rather than BMP signaling pathway. Instead of measuring the Smad4 level, other molecules can be measured, such as MITF (a regulator of cell differentiation), can be measured by western blot. For CR7, little information is known. To carry out further investigation, other factors which lead to cancer cell elimination can be tested. For example, the effects of ICAR on apoptosis, activating the immune system, or the effects on angiogenesis. To begin with, the structural change induced by ICAR can be tested in vivo. Inject ICAR in mice, after certain hours, take the ML cells out of the mice, and look at the structure using confocal microscopy.

The results (CR1 and CR5) suggest a positive relationship between the probabilities of cell differentiation and the decrease in cancer cell growth. This can be used to support the new dimensions of the hallmark of cancer. So oncological researchers can pay more attention to increasing the chance of cell differentiation. For the same purpose, the results (CR1 and CR2) suggested the direct relationship between the Smad4 level and the probability of cell differentiation. Although the research on the BMP signaling pathway is relatively mature, and many researchers have already been working on this pathway, it is still a new finding inside the ML cells. ML cells are different from normal cells in both genetic and phenolic properties. It is a valuable finding that the BMP signaling pathway also exists in ML cells and controls ML cell proliferation and differentiation.

All predicted results suggested a change in dosing (drug concentrations). As there is a positive response to those experiments, the dose-response curve should be produced. Then, comparing the ED50(effective dose), nH, and y-max parameters of the drugs on those tissues should help us to understand more about the drugs. To extend the assumption from the ICAR affecting on healthy cells, it is reasonable if the ED50 of drugs on the ML cells is much lower than that on the normal cells. So, the drug is still safe to use by controlling the daily dose. But to simplify the predictions, I assume that the ICAR does not affect controls.

In the investigation on the Smad4 levels, the durations of the drug on both cell lines were also measured. Looking at the durations would help us understand this drug's metabolism. This is an essential parameter for applying the drug to clinical research. As a cancer drug, the rate of metabolism for Smad4 cannot be too short (e.g., less than 1 hour), because patients cannot take the cancer drug too frequently. Also, it cannot be long as some substances might be dangerous if they remain in human bodies.

5. Conclusion

This investigation was carried out based on the previous study of the Chinese traditional medicine ICAR and the new direction in the hallmarks of cancer. By reading through the papers in that area, the hypothesis was conjectured as increasing the concentrations and treatment durations of Icariin increases the chance of myeloid leukemic (ML) cell differentiation by raising the Smad4 level and leading to inhibiting ML cell growth. Then, the three experiments were designed to prove this hypothesis (Western blot was used to measure the Smad4 level, the FACS was used to measure the CD157 level for differentiation, and the MTT was used to measure the tumor growth). The combination of results was imagined for those experiments, and the discussion and evaluation of those results were produced.

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