Dean&Francis
ISSN 2959-409X

MS003952

Ginsenoside Rb decreases the TSCC tumor cell viability and promotes apoptosis to decrease the tumor size by interacting with E-cadherin

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Abstract:

Ginsenoside Rd is a small molecule compound found in ginseng. In traditional Chinese medicine theory, it has anti-inflammatory, anti-edema, blood-activating, and debriding effects. In recent clinical trials, Rd has shown an effect of promoting cancer cell apoptosis, especially for oral cancer, with good curative effects. The specific mechanism of Rd treating cancer is not clear. Another recent study on the e-cadherin protein in cancer cells has given us a new research direction. ECAD is a major calcium-dependent adhesion molecule that is mainly found in cell adhesion in the cell and is usually considered an anti-cancer factor. However, recent research has shown that ECAD is crucial for maintaining cancer cell viability, and it can reduce the content of intracellular ROS by inhibiting TGF-β, P53, and other cellular pathways, thereby reducing the probability of cell death due to oxidative stress. In a series of experiments, a large reduction of ECAD resulted in increased cell apoptosis. This phenomenon is very similar to the effect of Rd on cancer cells. Therefore, this paper aims to study whether the mechanism of Rd treating cancer is related to E-cad.

Keywords: E-cadherin, Ginsenoside Rd, TSCC

1. Introduction

1.1 Background

Cancer is one of the deadliest diseases in the world. According to the cancer statistics from the American Cancer Society, there will be about 2 million new cases in the USA in 2024.[1] Even in today's highly advanced medical situation, certain cancers, such as lung cancer and breast cancer, still have a mortality rate of up to 15%. The basic mechanism of cancer is the uncontrolled proliferation of nonfunctional cells, taking the place of normal cells and breaking the organ's function.[2] Additionally, tumor cells usually have strong viability, fertility, and invasiveness.[3] These mutated cells are highly similar to the normal

ISSN 2959-409X

cells, which makes the treatment difficult.[4] They can evade immune detection and make the drug have to kill a large number of normal cells at the same time. This makes drug targeting extremely valuable in the treatment. Furthermore, because of this similarity, when we study the cellular pathways of tumor cells, we cannot only focus on whether they promote cell proliferation and invasion but also consider whether they affect cell viability and survivability.

Here, we will use Tongue squamous cell cancer (TSCC) cells as the sample. Tongue squamous cell carcinoma (TSCC) is an oral cancer with high malignancy and frequent early migration and invasion. This disease has a 5% mortality rate and is highly prone to causing disfigurement. Only a few drugs can treat tongue cancer: except the common chemotherapy and immunotherapy drugs for cancer, only two targeted drugs for TSCC are currently in use: Cetuximab and Pembrolizumab. Thus, Ginsenoside Rd is a new potential medicine in the treatment.[5]

Rd is a natural extract, also called Ginsenoside Rd. Ginsenoside are a class of natural product steroid glycosides and triterpene saponins. Compounds in this family are found only in the plant genus Panax (ginseng). As a class, Different types of ginsenosides exhibit vastly different clinical effects in the experiments.[6]

Some in vitro experiments have proved that Rd is capable of inhibiting tumor cell migration and promoting tumor cell apoptosis. However, we still lack in vivo test cases and clinical verification.[5]

CDH1 is a gene group. This gene encodes a classical cadherin of the cadherin superfamily. Mutations in this gene are correlated with gastric, breast, colorectal, thyroid, and ovarian cancer. Loss of function of this gene is thought to contribute to cancer progression by increasing proliferation, invasion, and/or metastasis. It is one of the most important cancer susceptibility genes. The ectodomain of this protein mediates bacterial adhesion to mammalian cells, and the cytoplasmic domain is required for internalization.[3] Usually, this gene is considered a typical tumor suppressor gene. However, recent research on human breast tumor cells showed that CDH1 has still been transcribed in multiple models of breast cancer. The main product, e-cadherin, also plays an important role in stabilizing tumor cell viability.

E-cadherin is the main protein that is transcribed by CDH1. The cadherin family is essential in maintaining cell-cell contact and regulating cytoskeletal complexes.[7] The structure of E-cadherin consists of 5 cadherin repeats (EC1 ~ EC5) in the extracellular domain, one transmembrane domain, and a highly phosphorylated intracellular domain. This region is vital to beta-catenin binding and, therefore, to E-cadherin function.

E-cadherin also has the function of mediating adhesion-dependent proliferation inhibition by triggering cell cycle exit via contact inhibition of proliferation (CIP) and recruitment of the Hippo pathway. It's a very important step in multiple cell migration, including angiogenesis and tissue invasion that is related to cancer. E-cadherin adhesions inhibit growth signals, which initiates a kinase cascade that excludes the transcription factor YAP from the nucleus. Conversely, decreasing cell density (decreasing cell-cell adhesion) or applying mechanical stretch to place E-cadherins under increased tension promotes cell cycle entry and YAP nuclear localization. In a word, e-cadherin plays an irreplaceable role in cell growth, migration, and death. In the tumor cells, e-cadherin is usually considered lacking or absent.[8][9]

TGFβ (transforming growth factor beta) is a multifunctional cytokine belonging to the transforming growth factor superfamily that includes three different mammalian isoforms (TGF-β 1 to 3, HGNC symbols TGFB1, TGFB2, TGFB3) and many other signaling proteins. TGFβ proteins are produced by all white blood cell lineages. TGFβ can bind to the TGFβ receptor on the cell surface to activate the pathway. The two main pathways that are led by TGFβ are the SMAD pathway and the DAXX pathway.[10]

P53, also known as tumor protein p53, cellular tumor antigen p53, or transformation-related protein 53 (TRP53), is a regulatory protein that is often mutated in human cancers. The p53 proteins are crucial in vertebrates, where they prevent cancer formation by their role in conserving stability by preventing genome mutation. So, p53 is classified as a tumor suppressor protein.[11]

ROS (reactive oxygen species) are highly reactive chemicals formed from diatomic oxygen (O2), water, and hydrogen peroxide. Some prominent ROS are hydroperoxide (O2H), superoxide (O2-), hydroxyl radical (OH), and singlet oxygen. ROS are pervasive because they are readily produced from O2, which is abundant. ROS can be a chemical signal in the cell. However, too much ROS may cause sober oxidative stress and lead to direct damage to cell organelles.

Till now, researchers have acknowledged that the tumor suppressor gene CDH1 can also play the role as an invasion suppressor, survival factor, and promoter of metastasis in breast tumor cells. The reason is that CDH1 can inhibit the ROS-producing pathways like TNFα, TGFβ, and P53. ROS and TGFβ are in direct cycle, ROS is not only the side product of TGFβ but also can enhance the TGFβ pathway. High ROS concentration will activate TNFα and P53 to start cell apoptosis. Additionally, ROS can also be the signal to activate some apoptosis or oxygen stress pathways like MAP3K and p38 pathways.[12]

The only flaw is the phenomenon that the amount of e-cadherin and ROS are in inverse proportion has only been proved and tested in breast cancer. So, in this experiment, I use the tumor cell from TSCC, which has a higher mortality and invasiveness. A stronger invasive tendency means that the cells are more susceptible to the appearance of e-cad. If the same phenomenon is found in TSCC cells as in breast cancer cells, it can be proven that e-cad is crucial for the survival of cancer cells.

For some Chinese medicine components like Rb, although there is a lot of clinical trial proof to show their effect on abnormal tissues like tumors, we still lack a comprehensive understanding of its mechanism. For example, there is still no evidence to show why it only causes the apoptosis of tumor cells and what molecules or pathways it actually affects.[13]

So, in this experiment, I predict that the mechanism of Rb to treat oral cancer has a relationship with the expression of e-cad. If this hypothesis can be confirmed, the treatment of cancer will have a completely new direction. And it is also greatly helpful to the development of herbal pharmacy. Even if the results show no relationship between the two, the results of the trial can still provide more cases for cancer research.

1.2 Hypothesis

The increasing concentrations and treatment durations with Rd decrease the expression of E-cadherin to decrease of tumor cell viability and increase apoptosis of SCC9 human tongue cancer, and decrease tumor size in SCC9 xenograft mice.

2. Method

2.1 CCK-8 test: Cell Proliferation and Cytotoxicity Assay

Seed SCC9 cells in a 96-well plate at a density of 10^4-10^5 cells/well in 100 μL of culture medium with or without compounds to be tested. Culture the cells in a CO_2 incubator at 37°C for 24 hours. Then Add 10 μl of 10μM, 20μM, 30μM, 40μM substances to be tested to the plate. Incubate the plate for an appropriate length of time (6, 12, 24 or 48 hours) in the incubator. Add 10 μL of CCK-8 solution to each well of the plate using a repeating pipette. Incubate the plate for 1 - 4 hours in the incubator. Mix it gently on an orbital shaker for 1 minute to ensure a homogeneous distribution of color. Then, read the plate. Measure the absorbance at 450 nm using a microplate reader. Repeat the experiment, but add 5 μl 100 μL Rb solution in the culture medium in the first step. Finally, repeat the whole experiment with 10 μl, 15μl, and 20μl 100 μL Rb solution. The experiments are done with MCE cell counting kit-8.

2.2 ANNEXIN V/PI FACS

To prepare the sample culture, first, induce apoptosis by adding 10μl different concentrations of Rb (5μM, 10μM, 15μM, 20μM) to SCC9 cell samples and culture them for 6h, and use another sample with only PBS as the negative control. Second, collect 2 x10^5 cells by centrifugation from each sample. Third, wash cells 1X with cold 1X PBS and carefully remove the supernatant. Fourth, re-suspend the cells in 1X Binding buffer at a concentration of ~1 × 10^6 cells/ml, preparing a sufficient volume to have 100 μL per sample. Finally, add 5 μl of Staining solution to tubes as indicated in table 1 below and gently swirl to mix.

To test the samples, first, incubate the mixture for 20 minutes at room temperature in the dark. Then add 400 μl 1X Binding buffer to each tube, gently mix or flick the tube, and analyze the cells immediately (within 1 hour) by flow cytometry.

Table 1. Cell Staining

Tube	Cells	Staining
1	Stabilized Control Cells	---
2	Stabilized Control Cells	5 μL Annexin V-FITC
3	Stabilized Control Cells	5 μL PI Solution
4	Un-induced Experimental Control	5 μL Annexin V-FITC + 5 μL PI
5	Apoptosis Induced Experimental Sample	5 μL Annexin V-FITC + 5 μL PI

2.3 Western blot

Whole-cell protein lysates were thawed on ice for 30 min before use. The amount of protein in each sample was quantified using a standard BCA assay kit. Equal protein amounts were loaded, with Laemmli sample buffer and β-mercaptoethanol, in 4–15% Mini-PROTEAN pre-cast gels. SDS–PAGE was performed at 200 V for 30–40 min, or until the dye front had just run off the gel. Gels were transferred onto PVDF membranes at 100 V for 1 h at

ISSN 2959-409X

4 °C. Membranes were then blocked with Odyssey blocking buffer for 1 h at room temperature. Primary antibodies were diluted at 1:1,000 into a 1:1 mixture of blocking buffer and TBS-T (TBS +0.2% Tween). Primary antibodies used in the study are SCC9 human cell e-cadherin antibodies. Samples were washed three times with 0.1% TBS-T and then incubated with secondary antibodies, diluted at 1:10,000, for 1 h at room temperature. Secondary antibodies used are anti-mouse IRDye 680RD (Licor, 925-68070). Samples were washed three times, for 5 min each, using 0.1% TBS-T. Samples were scanned using the Odyssey CLx imaging system.[12] The Western blot will be done three times.

2.4 Xenograft tumor model

Human oral cancer SCC9 cells are transplanted into the epithelial tissue of the tongue of five groups of SCC9 immunodeficient mice and kept for one month under the same conditions and on the same diet. The first group of mice was not given any treatment, while the second to fifth groups were orally administered 200μl of 50μM, 100μM, 150μM, and 200μM Rb solution, respectively, in every 12 hours. After one, two, three, four, and five weeks, the tumor will be measured and compared in size with caliper. The same experiment will be repeated 3 times.

2.5 Statistical analysis

Student's t-tests were used to determine differences between the groups. For experiment 4's xenograft test, all samples are compared with negative controls to generate data. The data are expressed as mean±SD. SPSS 16.0 (SPSS Inc., USA) was used for all statistical analyses. $P<0.05$ indicated statistical significance.

3. Results

Table 2. Experimental results and corresponding inferences

Combination Result # (CR#)					
	Rd decreases viability by CCK8?	Rd increase apoptosis by Annexin V/PI FACS?	Rd binds to ecadherin by affinity chromatography and western blot	Rd decreases xenograft tumor size by caliper	Support of hypothesis
1	+	+	+	+	Full
2	+	+	+	-	Partial
3	+	+	-	+	Partial
4	+	-	+	+	Partial
5	-	+	+	+	Partial
6	+	+	-	-	Partial
7	+	-	-	+	Partial
8	-	-	+	+	Partial
9	+	-	+	-	Partial
10	-	+	-	+	Partial
11	-	+	+	-	Partial
12	+	-	-	-	Partial
13	-	+	-	-	Partial
14	-	-	+	-	Partial
15	-	-	-	+	Partial
16	-	-	-	-	Fully Contradicts

Table legend: "+" indicates the phenomenons that are closer to the positive control, the positive control for test 1-3 is H2O2; "-" indicates the phenomenons that are closer to the negative control, the negative control for test 1-3 is PBS. For xenograft assay if the tumor becomes smaller, the result will be recorded as positive, or it will be recorded as negative.

CR1: If the experiment shows this result, it means that Rb can inhibit cell viability and promote the apoptosis of the SCC9 cells by interacting with e-cad, resulting in a de-

crease the tumor size.
CR2: If the experiment shows this result, it means that Rb can inhibit cell viability and promote the apoptosis of the SCC9 cells by interacting with e-cad but cannot decrease or cure the cancer effectively.
CR3: If the experiment shows this result, it means that Rb can inhibit cell viability, promote apoptosis, and decrease tumor size at some level but has nothing to do with e-cadherin.
CR4: If the experiment shows this result, it means that Rb may bind with e-cadherin and cause the tumor size and cell viability to decrease but not by promoting apoptosis.
CR5: If the experiment shows this result, it means that Rb promotes the apoptosis of SCC9 cells by interacting with e-cad and decreasing the tumor size, but the tumor cells won't lose their original viability because of this.
CR6: If the experiment shows this result, it means that Rb can inhibit the cell viability but not because of interact with e-cad and cannot decrease the tumor size.
CR7: If the experiment shows this result, it means that Rb can decrease the tumor size but not because the loss of e-cad promotes apoptosis.
CR8: If the experiment shows this result, it means that Rb can decrease the tumor size but won't influence the cell viability and apoptosis.
CR9: If the experiment shows this result, it means that Rb does decrease the cell viability, but the tumor won't be killed or cured by this.
CR10: If the experiment shows this result, it means that Rb promotes apoptosis and decreases the tumor size. But it won't bind with e-cad and decrease the viability.
CR11: If the experiment shows this result, it means that Rb can interact with e-cad and may induce cell apoptosis for this reason. But cells still have original viability, and the tumor keeps its size.
CR12: If the experiment shows this result, it means that Rb can decrease cell viability in some ways but not by interacting with e-cad. This is not enough to kill the tumor cells or decrease the tumor size.
CR13: If the experiment shows this result, it means that Rb can promote cell apoptosis but not by interacting with e-cad. Cells keep the viability, and the tumor keeps its size.
CR14: If the experiment shows this result, it means that Rb has a good affinity with e-cad, but that cannot really influence the tumor cells' viability and apoptosis. And the tumor keeps its size.
CR15: If the experiment shows this result, it means that Rb can decrease the tumor size but not by changing the cells' e-cad, viability, or apoptosis.
CR16: If the experiment shows this result, it means that Rb have no influence on tumor cells' viability, apoptosis. It also cannot decrease the tumor size or bind with e-cad.

4. Discussion

CR1: This result is completely in accord with the hypothesis, so it can prove that Rb does interact with e-cad to influence correlated ROS-produced signal pathways. This is able to cause cell apoptosis by surfeiting ROS and decreasing the tumor size. That means Rb can be an effective medicine for Tongue squamous cell carcinoma (TMCC) and a potential medicine for other cancers. From the research we can also see that Rb has a good affinity to the e-cad. And we can use this characteristic to treat early-stage cancer or as a carrier for other anti-cancer drugs.
CR2: This result shows that the interaction between Rb and e-cad can promote the apoptosis of tumor cells to some extent. However, it shows little effect on the decrease of the tumor size. This means it doesn't have the ability to prevent cancer cell colonization and cannot be a major medicine in the clinical treatment of cancer. But on the positive side, the ability to kill the isolated tumor cells may let it become a good medicine to decrease the tumor cells' number in the blood.
CR3: This result shows that the cell viability, apoptosis, and tumor size are all trending toward the positive control. That means Rb is most probably curing the cancer by decreasing the TSCC cell viability. However, the expression of e-cad does not change. So, it might work on other pathways or reduce the immune escape.
CR4: This result shows that the cell viability, inhibition of e-cad, and tumor size are all trending toward to positive control. So Rb might interact with e-cad and change the microenvironment to decrease the tumor size. The reason it doesn't kill cells in the in vitro test might be because it interacts with multiple pathways to kill the tumor cell. Or it may influence the immune evasion of the tumor cells.
CR5: This result shows that the cell apoptosis, inhibition of e-cad, and tumor size are trending toward to positive control. This means the cells are not dead because of low viability or aging. Some other apoptosis pathways, like the mitochondria lysis pathway, may cause this phenomenon. Or Rb has a natural virulence to the tumor cell that it breaks down directly.
CR6: This result shows that the cell viability and apoptosis are trending toward to positive control. The possible reason for this is the drug cannot work well in vivo tests. The tumor microenvironment may change the Rb structure, or the drug is lysis or pumped out directly by the drug resistance. Regarding the e-cad, we cannot make sure it has no relationship with Rb because it only tests its expression. If the Rb directly interacts with e-cad, the western blot will still show a negative result.

ISSN 2959-409X

CR7: This result shows that the cell viability and tumor size are trend toward to positive control. First, the Rb has little relationship with e-cad. Second, the reason why the tumor size decreases but the apoptosis has no change might be because Rb induces cell death beyond apoptosis. The possible explain is it breaks the immune evasion of the tumor cells. Since we cannot simulate an immune system in an in vivo environment, the in vivo test and the in vitro test show different phenomena.

CR8: This result shows that the inhibition of e-cad and tumor size are all trend toward to positive control. Because we don't have the data for the microenvironment change, we cannot assume that the lack of e-cad causes the tumor size to decrease. However, for an anti-cancer drug, this result is still valuable for research.

CR9: This result shows that the cell viability and inhibition of e-cad are trend toward to positive control. This means the lack of e-cad is highly possible to be the reason of viability decrease. However, this is not enough to kill the tumor cells. It may need higher concentrations, combined medicine, or only cause cell aging.

CR10: This result shows that the cell apoptosis and tumor size are trend toward to positive control. This shows Rb can kill the tumor cell and inhibit cancer effectively. However, the mechanism is not interacting with e-cad pathways. It will be worth testing TSG pathways in the future.

CR11: This result shows that the cell apoptosis and inhibition of e-cad are trend toward to positive control. So it can prove that the loss of e-cad can lead the cell apoptosis. However, the cell does not lose it original viability and the tumor size does not decrease. This might present that the e-cad is not the key signal and Rb does not work perfectly in an in vivo environment.

CR12: This result shows that only cell viability is trend toward to positive control. This means Rb works in different pathways with those in the hypothesis. But since it still can decrease the cell viability, it still a valuable research component in cancer treatment.

CR13: This result shows that only cell apoptosis trends toward the positive control. It might be because the in vitro environment does not have such a complex drug resistance system as that in vivo. So, Rb may kill the cell by some simple pathways in vitro. But in future tests, if Rb can keep the same function in vivo test, then it can be a potential drug to clear the free tumor cell in blood vessels and inhibit the invasiveness.

CR14: This result shows that only inhibition of e-cad is trend toward to positive control. Since it neither induces cell apoptosis nor decreases the tumor size. This result can be assumed as Rb is not a good medicine for cancer. But as we know the relationship between e-cad and the tumor cells, Rb can be a combined medicine.

CR15: This result shows that only tumor size is trending toward positive control. For the cancer treatment, this result is already sufficient to make Rb an anti-cancer medicine. And because this experiment still cannot explain the mechanism of Rb in this experiment, it still needs to take more tests to check out the actual pathways it interacts with.

CR16: This result shows that the cell viability, apoptosis, inhibition of e-cad, and tumor size are all trend toward to negative control. This means the hypothesis is not suitable to explain the phenomenon we observed. So, we can exclude the relationship between Rb, e-cad, and tumor apoptosis.

5. Conclusion

Through these possible experimental results, we can work out that Rb has an anti-cancer effect, Rb has pharmacological mechanisms related to E-cadherin and what effects it has on cancer cells, and Rb can be used as a drug to treat tongue cancer. Or whether it has some function as an adjunct to the treatment of cancer. Besides those results, the anti-tumor efficacy of Rb can be clearly seen. We can also analyze some partial results. For example, if Rb is only shown to promote apoptosis of cancer cells and does not decrease tumor size, we may use it to remove cancer cells from the blood. For future studies, we can also research whether Rb can inhibit other types of tumors and whether it has any side effects. Or to study the relationship between Rb and e-cadherin in more detail. It may explain why Rb shows a natural targeting in clinic tests.

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