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The potential inhibition effect of ellagic acid on liver cells in the treatment of hepatocellular carcinoma

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Abstract. Hepatocellular Carcinoma (HCC) is the most common kind of liver cancer, which has a relatively high fatality rate. The main cause of this type of cancer is liver inflammation, which leads to disorder in the tumor microenvironment. There are some current treatments, including liver transplants, radiofrequency, microwave ablation, and transarterial chemoembolization. However, the treatments still have limited disadvantages, which cannot be ignored. Recently, a traditional Chinese medicine - Chebulae Fructus, has been proven to have an inhibition ability on HCC cells. One of the main constituents, ellipticine, is found to have therapeutic targets that can treat HCC: PTGS2, CYP1A2, CCNB1, and RASGRF1. Moreover, the essay also mentioned that another type of constituent ellagic acid could also have an inhibition effect on tumor progression by binding to Cyclin-Dependent Kinase 6 (CDK6). This study will further investigate the HCC inhibition effect of ellagic acid by providing an overview of the predicted results of the experiment. It will also provide the possible research direction of ellagic acid HCC treatment.

Keywords: hepatocellular carcinoma, ellagic acid, xenograft, apoptosis.

1. Introduction

Liver cancer is the fifth most common cancer and the fourth leading cause of cancer-related deaths worldwide. [1]. The survivability of this cancer is relatively low (a three-year survival rate). Abdominal pain, inappetence, and jaundice are the relevant symptoms. HCC is cancer caused by inflammation, such as hepatitis B virus and hepatitis C virus with low responsiveness to chemotherapeutic agents [2]. After the unresolved inflammation remains long, the immune system will be stimulated to secret immune cells to repair the liver structure. However, infiltration of cells may cause the increased production of chemokine and cytokine, which is out of control. This leads to the risk of liver cell migration and, eventually, liver cancer [2]. The treatment of HCC includes surgical methods such as liver transplants. Chemical methods like radiofrequency, microwave ablation, and transarterial chemoembolization are also commonly used for liver cancer treatments [2]. Among them, liver transplant shows a relatively high curative effect with a 70% 5-year survival rate. However, the number of liver donors limits this kind of treatment. The tumor size and the number of nodules must also be considered [2]. What is worse, HCC patients are often diagnosed when they are in advanced stages, and many surgical treatments are of limited efficacy [3]. As for transarterial chemoembolization, the basis of the method is to obstruct the tumor arterial vessel to block the blood, oxygen, and nutrition supply to the tumor. At the same time, chemotherapeutic drugs are delivered, which can finally cause the death of the liver cancer cells [2].

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Nevertheless, transarterial chemoembolization has its disadvantages, too. The drug is not putting deeply enough into the tumor. It is also insufficient for not delivering the drug at an optimal clinical rate [4]. Sorafenib is the first-line targeted therapy for advanced HCC. The median overall survival (OS) of sorafenib monotherapy as first-line treatment for advanced HCC increased from 10.7 months (SHARP) to 14.7 months (CheckMate-459). The anti-tumor activity and safety of sorafenib have been validated in real-world settings. Noticeably, the application of sorafenib in clinical practice displays significant regional variations and complies with guidelines besides its usage as first-line therapy. Tremelimumab and durvalumab have also been used to break tolerance and stimulate T cells to release cytotoxicity against antitumor activity by inhibiting cellular pathways that down-regulate immune response. However, this treatment method also inevitably damages the human body, and the result of damaging tolerance may lead to normal tissue damage due to immunity [5]. Sorafenib is the first-line targeted therapy for advanced HCC. The median overall survival (OS) of sorafenib monotherapy as first-line treatment for advanced HCC increased from 10.7 months (SHARP) to 14.7 months (CheckMate-459) [6].

Recently, a traditional Chinese medicine Chebulae Fructus, has been proven to have an inhibitory effect on HCC cells [7]. The study used network pharmacology to reveal one of the active constituents of Chebulae Fructus, ellipticine. They also found several therapeutic targets of ellipticine that can treat HCC cells: PTGS2, CYP1A2, CCNB1, and RASGRF1. PTGS2 is an essential target for tumor cell proliferation, inflammation and invasion. The inhibition of this target will increase the mitophagy that is proceeding by PINK1-PRKN, which can further lead to the apoptosis of HCC cells. The knock-down of CCNB1 will inhibit the growth of HCC cells since CCNB1 is essential to this cancer's progression.

The study also mentioned that ellagic acid is one of the eight effective constituents of Chebulae Fructus. It has the highest binding affinity with Cyclin-Dependent Kinase 6 (CDK6), essential in tumor progression [7]. The therapeutic effects of Ellagic acid on cancer, hepatotoxicity, and fibrosis all indicate high pharmacological potential. It can prevent excessive proliferation of liver cells by regulating the outer membrane of mitochondria. Importantly, Japan has approved EA as an existing food additive, which demonstrates that EA can be used without toxicity restrictions [8]. Zhong mentioned that EA can significantly enhance the antitumor activity of DOX and DDP against hepatocellular carcinoma HepG2 and SMMC-7721 cells and reduce the cytotoxicity of DOX and DDP against normal liver HL-7702 cells. In nude mouse experiments, EA combined with relatively low dose DOX can effectively inhibit tumor growth and does not cause cardiactoxicity in high-dose DOX mice. They also found that ellagic acid's IC50 to normal liver cells is over 3-fold higher than in two HCC cell lines, revealing its selectivity of targeting cancer cells [b]. Another essay has mentioned that EA can be used in combination with other chemotherapeutic agents (DOX and DDP) to enhance the activity of chemotherapeutic agents against cancer cells (HepG2 and SMMC-7721) while effectively inhibiting their cytotoxicity against normal liver cells (HL-7702). This conclusion has also been verified in mouse experiments :EA combined with low-dose DOX can effectively inhibit tumor growth in mice, and does not produce cardiactoxicity[9]. This study is trying further to research the efficacy of ellagic acid in HCC treatment. The study uses ellagic acid to kill HepG2 cells in vitro and reduces tumor size in HepG2 xenograft mice. MTT assay and Annexin V and Pi test are used to measure the inhibition effect on HCC cells, just as the function seen on ellipticine.

2. Materials and Methods

2.1. Reagents

The positive control is taxol, and the negative control is DMSO solvent, which will be purchased from the company. Different concentrations of ellagic acid with 3μmol/L, 200 μmol/L, and 10 mmol/L will be tested on HepG2 cells with 12 hours, 48 hours, and 72 hours.

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2.2. Cell line

The HepG2 cell line was cultured in Dulbecco's Modified Eagle Medium (Gibco, USA) containing 10% fetal bovine serum at 37 °C with 5% CO2 and 95% humidity [2].

2.3. Annexin V and Pi test

The apoptosis trend of HepG2 cells will be measured by Annexin V and Pi test. After the designing duration time, Annexin V and PI working solution and the binding buffer were added. The media was incubated in the dark for 15 minutes, and then the final results can be seen by Flow Cytometry.

2.4. MTT assay

HepG2 cell viability can be detected by MTT assay. After the experiment, 20 μL MTT assay solution was added to the media, and the testing environment will be at a wavelength of 490 nm using a microplate reader [2].

2.5. Animal Study

8-10 immunodeficient mice were injected with HepG2 cell lines and injected subcutaneously in the flank of mice to set up the xenograft model [10].

3. Results

Table 1. The combination of possible results.

Possible results	Annexin V and PI tested by FACS increase?	cell viability of MTT assay decrease?	Mouse xenograft tumor size and shape decreases?	Support Hypothesis
CR 1	+	+	+	Yes
CR 2	+	+	-	Partially
CR 3	+	-	+	Partially
CR 4	-	+	+	Partially
CR 5	+	-	-	Partially
CR 6	-	+	-	Partially
CR 7	-	-	+	Partially
CR 8	-	-	-	No

Note: * "+" means a result that is the same or similar to the positive control taxol, and "- " means an impact that is the same or similar to the negative control PBS and DMSO.

Table 1 shows the proof of the inhibitory effect of ellagic acid on HCC through three experimental methods. A total of eight possible outcomes were summarized by combining different experimental phenomena from the three experiments.

3.1. Description of each combination

Combination of possible results 1 (CR1): the increase in Annexin V and PI results relevant to the cell apoptosis shows an apparent membrane integrity loss and apoptosis trend in HepG2 cells. MTT assay shows that the percentage of living cells is distinctly decreasing due to the inhibition effect of the ellagic acid. The tumor size of the xenograft mice also decreases compared with the negative control mice.

Combination of Possible results 2 (CR2): in this kind of result, although both the Annexin V and Pi test and the MTT assay shows positive results with the increasing number of apoptosis and dead cells, the size of the tumor cell on xenograft mice does not show significant change. The results indicate that although ellagic acid can inhibit tumor progression, the inhibition is not effective enough to apply the constituent to medical use directly. Some adjuvant drugs should combine with the current ellagic acid to become an efficient drug supply.

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Combination of Possible results 3 (CR3): For result 3, the Annexin V and Pi test and the xenograft tumor size both show positive results with the increasing apoptosis cell trend. However, the MTT assay graph has little change compared to the negative control PMS. Due to this result, it is reasonable to infer that ellagic acid causes HepG2 cell damage to a certain degree. Still, it does not kill the cell eventually, which may have the risk of cancer recurrence in actual medical treatment.

Combination of Possible results 4 (CR4): This test shows a relatively solid pharmaceutical effect refers to the result. MTT assay and the xenograft tumor size prove that ellagic acid kills the HepG2 cells directly. However, the Annexin V and Pi test shows that no cells remain in the apoptosis period. It means that ellagic acid has substantial toxicity to the HepG2 cells. For this result, the toxicity of ellagic acid needs to be further researched to avoid harm to human body cells.

Combination of Possible results 5 (CR5): Results 5 show that only Annexin V and Pi test shows positive results. Hence, the ellagic acid only causes the apoptosis of HepG2 cells but does not kill the cell or have medical application value individually. The ellagic acid can be used as the adjuvant drug with other methods as a common treatment for HCC.

Combination of Possible results 6 and 7 (CR6 and CR7): The results of tests 6 and 7 may exist because of the constituent decomposition in the organism. In result 6, the MTT assay is positive, but Annexin V and Pi and the xenograft tumor size show a negative result. It means that although no cell is experiencing apoptosis and the size does not change, ellagic acid kills HepG2 cells. For the result in test 7, Annexin V and Pi test and MTT assay are negative results. However, the size of the tumor cell is decreasing, which indicates that although ellagic acid neither causes damage to the cell nor kills the cell, the tumor size is changing small.

Combination of Possible results 8 (CR8): The result of test 8 is close to the negative control. All three tests, Annexin V and Pi, MTT assay, and xenograft tumor size, do not show positive results. The result proves that the ellagic acid does not have effective positive effects compared to the PBS. This may happen if no suitable targets on HepG2 for ellagic acid exist.

4. Conclusion

According to the predicted results of these tests, we can see an overview of the possible inhibition effect of ellagic acid on HCC cells. Combination 1 is the ideal result that can kill the HCC cells, stimulating apoptosis and decreasing xenograft tumors. It is proved that EA has effective anti-cancer properties, and the side effects on human cells are reduced as much as possible. The excessive proliferation of cancer cells is fully inhibited. It is an ingredient that can fully protect the treatment effect of cancer patients and reduce the harm to the human body.

Combinations 2-4 partially suit the hypothesis with one negative factor, respectively. They show the distinct effects that ellagic acid has on HepG2. However, they also prove that the mechanism and the final results differ from the taxols. For these results, further research should be processed to solve whether any side effect or improvement can be made for ellagic acid application for medical use.

Combination 5 shows a less effective result on ellagic acid for only Annexin V, and Pi test has a positive result. Although it is possible to speculate that EA has few drug side effects on normal liver vell line, it also indicates that EA cannot be used as a single therapeutic agent to adequately extend the survival time of HCC patients. Then ellagic acid is better to be an adjuvant drug with the combination of other drugs on HCC treatment.

Combinations 6-7 are the experiment results that are hard to infer since they may not suit the logic of the inhibition effect. However, it may also be that the natural breakdown of drugs by cells leads to the minimization of the effect of drugs on cells. Hence, there is no valid data to support their effects in cell apoptosis experiments. Also, since the tests still need to be done, the two results cannot be excluded directly.

Combination 8 opposes the hypothesis. It shows that ellagic acid is not a suitable drug for HCC treatment since both three factors in the test show negative results. HepG2 cell growth is not affected by this constituent. However, the possibility is minimal based on the data found. A study has shown that EA can bind to DNA to form potent adducts that block carcinogens in the liver[c].

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The concentration degree and the duration time also need to be considered when predicting the results. In the overall combination of possible results, the cell apoptosis trend is proportional to the increasing concentration of the ellagic acid, which indicates that the inhibition effect is more substantial when the amount of the ellagic acid increases. The different duration period reveals that the xenograft tumor size is decreasing level also increases with the longer extension of the experiment time.

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