Rosmarinic acid inhibits inflammation and angiogenesis of hepatocellular carcinoma by suppression of NF-κB signaling in H22 tumor-bearing mice

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Abstract

The aim of this study was to explore the anti-tumor effect and therapeutic potential of rosmarinic acid (RA) in the treatment of hepatocellular carcinoma (HCC). RA at 75, 150 and 300 mg/kg was given to H22 tumor-bearing mice by intragastric administration once daily for 10 consecutive days. Levels of inflammatory and angiogenic factors, including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β) were measured by enzyme linked immunosorbent assays (ELISA). Protein levels of phosphorylated NF-κB p65 and p65 were detected by western blot. mRNA level of NF-κB p65 was analyzed by qRT-PCR. The results showed that RA could effectively suppress tumor growth with fewer toxic effects by regulating the secretion of cytokines associated with inflammation and angiogenesis, and suppressing the expression of NF-κB p65 in the xenograft microenvironment. Our findings unveil the possible anti-tumor mechanisms of RA and support RA as a potential drug for the treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer related mortality worldwide and it is still an intractable problem though several synthetic drugs are available for HCC clinical treatment. These synthetic drugs have low selectivity and often cause serious adverse reactions. Therefore, the development of novel drugs with higher efficiency and fewer side effects is imperative.

Rosmarinic acid (RA; α-caffeoyl-3,4-dihydroxyphenyl lactic acid) is a natural phenolic compound that exists in and thus can be extracted from many medicinal species of Boraginaceae and Lamiaceae, including rosemary, mint, perilla frutescens and sarcandra glabra. It has been reported that RA exerts a variety of pharmacological activity, such as antioxidant, anti-angiogenic, anti-inflammatory, anti-fibrosis, and hepatoprotective. These effects may be related to its phenolic hydroxyl group. Furthermore, recent studies have revealed that RA has antineoplastic activity in gastric carcinoma, colon carcinogenesis and leukemia. Han et al. investigated the effect of RA on MKN45 human gastric cancer cells and found that RA exerted an anti-cancer effect via the inhibition of pro-inflammatory cytokines and inactivation of the inflammatory pathway. Venkatachalam et al. analyzed the antineoplastic activity of RA against 1,2-dimethylhydrazine induced rat colon carcinogenesis and found that RA reversed the decrease of antioxidant status and the elevation of CYP450 content. Moon et al. reported that RA treatment sensitizes TNF-α-induced apoptosis in human leukemia U937 cells through the suppression of nuclear factor-κB (NF-κB) and reactive oxygen species (ROS). The above reports are several of the previous studies on the anti-cancer effects of RA. However, its potential on HCC therapy has rarely been explored.

The investigation of inflammation-associated carcinogenic process in HCC is the most extensive. Epidemiologic study showed that more than 90% of HCC reports are involved in inflammation. NF-κB transcription factor, a modulator of cell growth, immune responses and inflammation, is one of the most important signal pathways and has been found to be constitutively active during inflammation and carcinogenesis of liver. The heterodimer p65 is a representative form of NF-κB. A key role of NF-κB in...
liver homeostasis was first reported by studying p65 knockout mice (14). Activation of NF-κB p65 increases the levels of various cytokines in tumor microenvironment, thereby enhancing tumor growth (15, 16). Recent studies have also demonstrated that NF-κB p65 is involved in angiogenesis by regulating angiogenesis factors during HCC development (17). Therefore, NF-κB p65 is a promising target for the treatment of HCC, in which targeting both the tumor microenvironment and angiogenesis may be of importance. In the present work, the H22 hepatocarcinoma xenograft tumor model was established in mice to study the role of NF-κB p65 signaling pathway mediated by RA in HCC and explore possible molecular mechanisms related to inflammation and angiogenesis in the xenograft microenvironment.

2. Materials and methods

2.1. H22 hepatocarcinoma xenograft tumor establishment in mice

SPF male Kunming mice weighing ranging from 18 to 22 g were purchased from Experimental Animal Center of Guangxi Medical University (Guangxi, China). The H22 hepatocarcinoma cell lines were offered by Nanjing KeyGEN Technology Development. All animal experimental procedures in this study were conducted according to protocols approved by the institutional ethical committee of Guangxi Medical University. The feeding conditions were as follow: temperature 25 ± 2 °C, humidity 60 ± 10%, and illumination time 12 h. After 7 days injection of H22 cells into the abdominal cavity of Kunming mice, ascites was extracted from the tumor-bearing mice and was diluted to the concentration of 1 × 10^7/ml with PBS. Then, 0.2 ml of the cell suspension was inoculated subcutaneously into the right side of the axillary of each mouse. After inoculation for 24 h, the tumor-bearing mice were randomly divided into 5 groups of 8 mice each. A group of mice without tumor inoculation was also included as non-tumor group. The non-tumor and model group received the same amount of normal saline. The positive control group received cyclophosphamide (CTX, Shaxni Powerdome Pharmaceuticals Co., Ltd., Shaxni, China) at a dosage of 20 mg/kg once every two days via intraperitoneal injection. The low-, middle- and high-dose RA group received rosmarinic acid diluted in normal saline (purity > 98%, Chengdu must bio-technology Co., Ltd., Chengdu, China) at different dosages (75, 150 and 300 mg/kg) once a day by intragastric administration, respectively. All animals were euthanized after 10 days treatment. The blood samples were collected from the eyeballs of mice. Xenograft tumors and corresponding organs (liver, thymus, and spleen) were collected and weighed.

The tumor growth inhibition rate and organ index were calculated by the formula described below:

\[ \text{Tumor growth inhibition rate} \% = \left( \frac{W_{\text{Control}} - W_{\text{Treated}}}{W_{\text{Control}}} \right) \times 100 \]

\[ (W_{\text{Model}}: \text{mean tumor weight of model mice}; W_{\text{Treated}}: \text{mean tumor weight of treated mice}) \]

\[ \text{Organ index} = \text{organ weight (mg)/body weight (g)}. \]

2.2. Analysis of serum physiochemical indexes

Serums were extracted from blood samples by centrifugation at 3000 g for 10 min. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), uric acid (UA) and creatinine (CRE) were determined by a HITACHI7100 automatic biochemical analyzer (HITACHI, Japan) according to the instructions of the manufacturer.

2.3. Pathological observation

The tumor samples of each group were removed and fixed with buffered neutral 10% formalin, embedded in paraffin, and sliced in 5 μm thickness with a rotary microtome. The slices were stained with hematoxylin eosin (HE) and photographed under an optical microscope. The photographs were taken at > 400 magnification.

2.4. ELISA measurements of cytokines in xenografts tumor

Xenograft tumor tissues were washed and then homogenized on ice with normal saline. Homogenates were centrifuged at 3000 g for 10 min at 4 °C and the supernatants (100 μl) were used for analysis. Levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β) were measured by enzyme linked immunosorbent assays (ELISA) kits (Ebioscience, USA) in triplicate according to manufacturer’s recommended protocol.

2.5. Western blot

Xenograft tumor tissues were lysed on ice in RIPA lysis buffer (Beyotime institute of biotechnology, Jiangsu, China) for 10 min. The lysates were centrifuged at 14,000 g for 10 min at 4 °C and supernatants were collected. The proteins were quantified with a BCA Protein Assay Kit (BOSTER, Wuhan, China). Equal amount of proteins (40 μg) was loaded onto 12% SDS-PAGE and transferred to PVDF membranes (Millipore Corporation, USA). The membranes were blocked in 5% non-fat dried milk buffered for 1 h and incubated with primary antibodies against phosphorylated p65 (Ser536) (1:1000) and p65 (1:1000) (Bioworld, USA) overnight at 4 °C. Then the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, USA) for 2 h at room temperature. Blots were washed three times with tris buffered saline with tween-20 (TBST) and detected by an odyssey infrared imaging system (LI-COR, USA) following the manufacturer’s instructions. The quantification was normalized with the corresponding value of β-actin expression.

2.6. RNA extraction and quantitative real-time PCR

Total RNA was extracted from the tumor samples by Trizol Reagent (Invitrogen, USA) according to the manufacturer’s instructions. Total RNA (2 μg) was reverse transcribed to cDNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). Quantitative real-time PCR was performed by Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA) following the conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The primer sequences are listed as follows: NF-κB p65 (forward, TGTGGCAACAGTGGCAAAA; reverse, TTGAGTTCCGGTGAGGCA), and GAPDH (forward, GTCTGAG-TATGCTGGAGT; reverse, ATGGGGTGGAGAACAGG).

2.7. Statistical analysis

All data were presented as mean ± SD. Statistical analysis was performed with SPSS 16.0 software. The significance of the data was determined by one-way analysis of variance (ANOVA) followed by Dunnett's t-test, and P < 0.05 was considered statistically different.
3. Results

3.1. Effects of RA on H22 xenograft tumor growth

After 10 days treatment, one of the tumor-bearing mice in model group was dead. Mice in the treatment groups were all survived and the survival ratio was 100%. The tumor and body weights of mice in each group were also measured (Table 1). We found that the positive control group and three dosage RA groups showed marked inhibition in the growth of the H22-xenograft tumor. The average tumor weight of model group was 2.28 ± 0.63 g. Compared with the model group, the treatment of CTX and 75, 150, and 300 mg/kg RA resulted in the decreased average tumor weights of 0.84 ± 0.39, 1.39 ± 0.40, 1.30 ± 0.74, and 1.18 ± 0.42 g, respectively ($P < 0.05$, $P < 0.01$). Thus, the tumor inhibition rates of the positive control group and RA (75, 150, and 300 mg/kg) groups were found to be 63.15%, 39.03%, 42.98% and 48.24%, respectively. These results suggested that the treatment of CTX and 300 mg/kg RA could effectively inhibit the tumor growth in H22 tumor-bearing mice. But CTX can inhibit the increase in the average body weight of tumor-bearing mice compared with model group ($P < 0.05$), whereas no significant loss of weight was found among the RA groups.

HE staining of the tumor tissues was observed at ×400 magnification to evaluate the pathological changes of each group (Fig. 1). The results showed that tumor cells of model group were increased in number and volume, exhibited an even distribution, significantly increased nuclear volume, inflammation, and vascular proliferation. Compared to the model group, the tumor cells of CTX and RA groups were decreased in varying degrees. The pathological results of high-dose RA group and CTX group revealed that the tumor cells were broken and the percentage of apoptotic cells was increased, which indicated that these can suppress growth and development of tumor cells effectively.

3.2. Toxicological effects of RA on tumor-bearing mice

In anti-cancer treatment, it could be a challenge to trigger tumor cell death without affecting other normal organs. To explore the toxicological impacts of RA on tumor-bearing mice, the thymus, spleen, and liver indexes were measured. The results showed that RA had little impacts on thymus, spleen and liver indexes (Fig. 2). Moreover, the liver and renal function parameters including ALT, AST, BUN, UA, and CRE were also measured. The levels of the renal function parameters remained unchanged after the treatment of RA whereas levels of AST were increased (Table 2). The increased levels of AST may be related to the H22 hepatocarcinoma xenograft tumor model.

3.3. Effects of RA on cytokine levels in xenograft tumor

The effects of RA on the levels of IL-1β, IL-6, TNF-α, VEGF, and TGF-β in tumor tissues were analyzed by ELISA. As shown in Fig. 3, the treatment of CTX and 300 mg/kg RA significantly decreased the elevation of all these cytokines compared with those in model group ($P < 0.05$, $P < 0.01$). For IL-1β, TNF-α and TGF-β, the middle-dose RA group also performed obvious inhibitory effects on the levels of IL-1β, TNF-α and TGF-β ($P < 0.01$).

3.4. Effect of RA on NF-κB p65 signaling in xenograft tumor

The tumor microenvironment plays a vital role in development and progression of cancer. To assess the effects of RA on NF-κB p65 on the xenograft microenvironment in H22 tumor-bearing mice, protein expressions of p65 and p-p65 were detected by western blot and mRNA levels of p65 were measured by qRT-PCR in xenograft tumors. Thus, the protein and mRNA expressions in these analyses represent the expression of mouse cells infiltrating or surrounding the xenografts. Phosphorylation of p65 plays an important role in modulating NF-κB activity. Western blot analysis showed that the CTX group and three RA groups decreased p65 phosphorylation (Fig. 4A and B, $P < 0.01$). qRT-PCR results showed that the mRNA levels of p65 were decreased after CTX and 150 mg/kg RA treatment compared with model group (Fig. 4C, $P < 0.05$).

4. Discussion

Previous studies have been reported the protective effect of RA on liver fibrosis (18) and liver injury (8) due to its antioxidant and anti-inflammatory properties. But its antineoplastic activities in liver cancer are rarely reported. At present, more and more concern is being focused on investigating the underlying mechanisms involved in HCC and new potential drugs in animal models. In the present study, the H22 hepatocarcinoma xenograft tumor model was established in mice to study anti-tumor effects and possible molecular mechanisms of RA in HCC. The results showed that after 10 days treatment with RA, only one mouse in model group was dead, and mice in the treatment groups were all survived. The average tumor weights of H22 tumor-bearing mice were significantly decreased compared with model group. In addition, pathological examination also demonstrated that RA could suppress tumor cells growth and vascular proliferation. Thymus and spleen are essential immune organs of the host animals. In this study, CTX was used as the positive control drug. Results showed that the CTX group can inhibit the tumor growth more effectively than RA groups. However, it dramatically decreased the thymus and spleen organ index, as well as the body weights of tumor-bearing mice compared with model group, whereas RA had little influence on the immune indexes and body weights. Moreover, RA showed little impact on the liver index and renal function parameters. These data suggested that RA could effectively inhibit the tumor growth with fewer toxic effects and could be considered as a promising therapeutic agent in the treatment of HCC.

The functional relationship between inflammation and cancer has been extensively studied. An inflammatory microenvironment

![Table 1](image)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dosage (mg·kg⁻¹)</th>
<th>Body weight (g)</th>
<th>Increase of body weight (g)</th>
<th>Tumor weight (g)</th>
<th>Average inhibition rate (%)</th>
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<td></td>
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<td>Pre-treatment</td>
<td>Post-treatment</td>
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<tr>
<td>Model</td>
<td>—</td>
<td>20.34 ± 1.16</td>
<td>33.75 ± 1.94</td>
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<td>32.23 ± 2.70</td>
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<td>10.59 ± 1.06</td>
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Model group (n = 7), CTX and RA groups (n = 8); *P < 0.05 vs model group, **P < 0.01 vs model group.
Fig. 1. Pathological results of H22 xenograft tumor tissues after 10 days treatment with RA (HE, magnification ×400). Compared with the model group, the number of tumor cells in the CTX and RA groups was decreased in varying degrees.

Fig. 2. Effects of RA on thymus, spleen and liver indexes. Mice were treated according to the procedures described following the methods. After the last administration, thymus, spleen and liver of the mice were collected and weighed. Organ index was measured according to the above formula. Data were presented as mean ± SD. *P < 0.05 compared with model group.
is considered to play a vital role in different stages of HCC development (19). However, until now, it is still a significant challenge that inflammation promotes tumor growth and how inflammation interacts with tumorigenesis. Even so, one fact is that inflammation does influence every step of tumor development, including tumor initiation, angiogenesis, invasion and metastasis (20). During the initial stage of tumor progress, the inflammatory environment can promote mutated cells proliferation (21). Furthermore, tumor promotion triggered by inflammation might occur early or late during tumorigenesis through overproduction of pro-inflammatory cytokines secreted by immune or inflammatory cells (22,23).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dosage (mg·kg⁻¹)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>BUN (g/dL)</th>
<th>UA (mg/dL)</th>
<th>CRE (mg/dL)</th>
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<td>Non-tumor</td>
<td>—</td>
<td>67.40 ± 10.60</td>
<td>113.4 ± 13.56</td>
<td>8.62 ± 0.98</td>
<td>42.00 ± 14.71</td>
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<td>78.14 ± 15.00</td>
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<tr>
<td>CTX</td>
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<tr>
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<td>75</td>
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<td>484.0 ± 113.5</td>
<td>5.13 ± 0.39</td>
<td>53.00 ± 5.57</td>
<td>14.00 ± 3.00</td>
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<td>150</td>
<td>85.33 ± 17.67</td>
<td>447.0 ± 150.6</td>
<td>5.24 ± 0.62</td>
<td>44.33 ± 9.07</td>
<td>12.67 ± 1.53</td>
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<td>300</td>
<td>89.00 ± 9.64</td>
<td>742.7 ± 99.52**</td>
<td>5.20 ± 0.55</td>
<td>52.33 ± 5.03</td>
<td>16.33 ± 2.31</td>
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Table 2: Effects of RA on serum indexes of hepatic and renal functions in H22 tumor-bearing mice (mean ± SD).

Model group (n = 7), CTX and RA groups (n = 8); *P < 0.05 vs model group, **P < 0.01 vs model group.

Fig. 3: Effects of RA on cytokine levels. Xenograft tumor tissues were collected after RA treatment. Levels of IL-1β, IL-6, TNF-α, VEGF, and TGF-β in tumor tissues were measured by ELISA. Data were presented as mean ± SD. *P < 0.05 compared with model group, **P < 0.01 compared with model group.
NF-κB is a major intrinsic pathway that mediates inflammation and tumorigenesis. Phosphorylation of p65 is an important mechanism modulating NF-κB activity. Many stimuli can induce NF-κB activation, including the overproduction of many cytokines. Constitutive NF-κB activation can lead to increased levels of certain pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. These pro-inflammatory factors are powerful cytokines in NF-κB activation (24). Therefore, it is generally believed that NF-κB and inflammation form a positive feedback loop (19). In this study, we found that RA can not only decrease the overproduction of IL-1β, IL-6, and TNF-α but also suppress the expressions of p65 and p-p65 compared with model group in the xenograft microenvironment. These data suggested that RA has an important effect on the expression of NF-κB p65 and the overproduction of cytokines in the tumor microenvironment. Moreover, qRT-PCR result showed that the mRNA level of NF-κB p65 was also decreased after RA treatment, suggesting that RA can decline the transcription and translation of p65 in H22 tumor-bearing mice.

HCC is considered as a vascular-dependent malignant tumor and angiogenesis is related to the tumor growth and metastasis (25). Recent studies revealed that NF-κB p65 is also involved in angiogenesis by regulating key angiogenesis factors such as VEGF, TGF-β, IL-6, and TNF-α (26,27). VEGF, as a pro-angiogenic factor, is a major regulator of angiogenesis in tumor progression and the expression of VEGF is triggered by growth factors, oncogenes and hypoxia (28). TGF-β is another important factor in adjusting neovascularization and act as a tumor promotor in advanced cancer. Previous studies have shown that TGF-β is over-expressed in HCC and NF-κB is closely related to TGF-β1 expression (29). Additionally, IL-6 and TNF-α, known as inflammatory cytokines, are also well-recognized pro-angiogenic factors which can accelerate angiogenesis and vascular remodeling (30,31). Results of the current study showed that RA can suppress the secretion of VEGF, TGF-β, IL-6, and TNF-α, suggesting that RA exerts anti-tumor effects through inhibition of angiogenic factors. The underlying mechanisms may be, at least partly, via inhibition of NF-κB p65.

In conclusion, these studies demonstrated that RA could effectively suppress the tumor growth in H22-xenografts model with fewer toxic effects. The mechanisms involved in the anti-tumor effect of RA on HCC were associated with inhibition of inflammatory cytokines and angiogenic factors, as well as suppression of NF-κB p65 in the xenograft microenvironment. The tumor microenvironment might be a key target for the treatment of liver cancer. These results may provide new insight into the anti-tumor mechanisms of RA in HCC. Based on our data, we believe that RA may be a potential medicine for drug development against HCC although more studies are still needed to explore the underlying mechanisms.

Conflict of interest

The authors declare that they have no conflict of interests.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (81260511).

References
