

Inhibitory Effect of Ethyl Pyruvate on Orthotopic Transplantation of Gastric Cancer in Severe Combined Immunodeficiency Mice

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Abstract: *Objective:* To investigate the inhibitory effect and mechanism of ethyl pyruvate (EP) on the growth and liver metastasis of orthotopically transplanted gastric cancer in severe combined immunodeficiency (SCID) mice. *Methods:* SCID mice were orthotopically transplanted with SGC-7901 human gastric cancer tissue to establish a liver metastasis model of gastric cancer. Animals were injected intraperitoneally with different concentrations of EP. After 30 days, gastric cancer and metastatic liver tissues were taken out to detect the volume and weight of gastric cancer tissues and the number of metastatic liver nodules. Real-time quantitative PCR and immunohistochemistry were used to detect high mobility group protein B in different groups. Expression levels of 1 (HMGB1), receptor glycation end product receptor (RAGE), NF- κ B, vascular endothelial growth factor (VEGF), and membrane type 1 matrix metalloproteinase (MT1-MMP). *Results:* Compared with the control group, the weight and size of gastric cancer tissue and the number of metastatic liver nodules in the EP treatment group were significantly reduced ($P < 0.01$). EP inhibited the expression of HMGB1, RAGE, VEGF and MT1-MMP in gastric cancer and metastatic liver tissue, but had no significant effect on NF- κ B expression. *Conclusion:* EP may inhibit the growth of gastric cancer and liver metastasis in SCID mice by down-regulating HMGB1-RAGE pathway, which may have therapeutic effects on cancer.

Keywords: Severe Combined Immunodeficiency, Orthotopic Transplantation, Gastric Neoplasms, Tumor Metastasis

1. Background

Malignant gastric tumors originate from the most epithelial mucosal epithelial cells in the stomach wall, which can occur in various parts of the stomach (the most pyloric pyloric region, the gastric fundus sacral area, and the stomach body slightly less), which can invade the different depths and breadths of the stomach wall. Cancerous lesions localized in the mucosa or submucosa are called early gastric cancer, and those who violate the muscular layer or have metastasis to areas outside the stomach are called advanced gastric cancer [1-2]. There are many forms of gastric cancer observed by the naked eye or gastroscopically, such as superficial, mass, ulcer, invasive, and ulcerative cancer. Microscopically, there are many types of cancer cells, such as adenocarcinoma,

adenosquamous carcinoma, squamous cell carcinoma, undifferentiated carcinoma, and carcinoid. The molecular structure inside the finer cancer cells is also very different. Therefore, although it is called gastric cancer, even if the types seen under the naked eye and the microscope are the same, the personality is still very different [3-4]. Gastric cancer is one of the most common malignant tumors in the world, and mortality ranks second in malignant tumors. The main cause of death in gastric cancer patients is tumor invasion and metastasis [5]. Therefore, the inhibition strategy of exploring targets related to gastric cancer metastasis has important research significance. Studies have shown that the HMGB1-RAGE pathway plays an important role in the

development of tumors, which can promote the proliferation of tumor cells and block the apoptosis of cells [6]. As a potent inhibitor of high mobility group protein B-1 (HMGB1), ethyl pyruvate (EP) not only has anti-inflammatory activity, but also inhibits the release of HMGB1 in tumor cells and induces apoptosis of tumor cells [7].

Therefore, this study aimed to investigate the inhibitory effect and molecular mechanism of EP on the growth and liver metastasis of orthotopically transplanted gastric cancer in severe combined immunodeficiency (SCID) mice, and provide new methods and strategies for the treatment of gastric cancer.

2. Materials and Methods

2.1. Materials

EP and its diluent Lactated Ringer's solution was purchased from Beijing Ruixianghe Biotechnology Co., Ltd., PCR primer and dsDNA Oligo were synthesized by Shanghai Jianglai Biotechnology Co., Ltd., DMEM medium, fetal bovine serum and trypsin were purchased from Fermentas, USA. HMGB1, receptor glycosylation end product receptor (RAGE), NF- κ B, vascular endothelial growth factor (VEGF) and membrane type 1 matrix protease (MT1-MMP) antibodies were purchased from Amresco, USA, gastric cancer SGC-7901 The cell line was purchased from Bo Yan (Shanghai) Biotechnology Co., Ltd., and SCID female mice (7 weeks old, body weight 22 ± 2 g) were purchased from the Experimental Animal Science Department of Capital Medical University.

2.2. Animal Model

Thirty SCID mice were randomly divided into control group (n=10), EP 50 mg/kg group (n=10) and EP 100 mg/kg group (n=10). Animal model preparation was performed by subcutaneous transplantation of SGC-7901 human gastric cancer tissue in nude mice. After the animal was anesthetized, the left upper abdomen skin, abdominal wall and peritoneum were dissected, and the stomach wall was exposed. In the middle of the large curvature of the stomach, the gastric serosal membrane was damaged by scissors for about 5 mm, and the human gastric cancer tissue block (100 mg, about 10mm³ size) was sewn with a No. 0 silk thread. At the site of injury, the stomach is then delivered into the abdominal cavity, and the skin of the abdominal wall is sutured with silk thread.

2.3. Animal Experiment Methods

After the animal model was prepared, EP 50 mg/kg and EP 100 mg/kg were treated as intraperitoneal injections of SCID mice, once a day for 30 days. An equal volume of EP dilution was also injected intraperitoneally into SCID mice as a control group once daily for 30 days. The length and short diameter of the tumor, the weight and the number of metastatic liver nodules were measured every 2d, where the tumor volume = (long diameter \times short diameter ²)/2.

2.4. Specimen Collection and Pathological Examination

The mice were sacrificed 30 days after treatment. The gastric cancer tissues and metastatic liver tumor tissues were taken, fixed with 5% formaldehyde, embedded in paraffin, and pathological sections were made. HE staining was performed to observe the tumor metastasis. At the same time, some gastric cancer tissues were preserved under liquid nitrogen.

2.5. Real-Time Quantitative PCR

The total RNA of gastric cancer and metastatic liver tumors in EP 50mg/kg group, EP 100mg/kg group and control group were extracted and reverse transcribed into cDNA. The upstream sequence of HMGB1 primer: 5'-GACAGGCAAAGATATAAGCG-3'; downstream sequence: 5'-CTCCTCAGGATTAGGCTGTT-3'; upstream sequence of RAGE primer: 5'-CCCAACATGGAAGTGTACTG-3'; downstream sequence: 5'-CTGTCTCCTTCGGTTCTGAT-3'; NF- κ B primer upstream sequence: 5'-TGTGGCTGGGTCACGTATC-3'; downstream sequence: 5'-CGTCAACAACCAGATCAACT-3'; VEGF primer upstream sequence: 5'-TAGAGAGAAGCAGCCAGCAG-3'; downstream sequence: 5'-TTTCCCTTCCTCGAACTGA-3'; MT1-MMP primer upstream sequence: 5'-CACACTGAGTCGTCAATGGC-3'; downstream sequence: 5'-GGAATGGGTAGGAAGGAAGT-3'; GAPDH primer upstream sequence: 5'-AGCACAATTGGCGAATCTAC-3'; downstream sequence: 5'-GCATGGTCTACTGAAGGGCA-3'. PCR amplification was performed using the above primers on a real-time PCR instrument with GAPDH as a reference. Amplification system 20 μ l: SYBR Green 10 μ l, ROX 1 μ l, ddH₂O 5 μ l, cDNA 2 μ l, upstream primer 1 μ l, downstream primer 1 μ l. Reaction conditions: first step: 92°C 30 s, second step: 92°C 45 s, 40°C 50 s, third step: 92°C 30 s, 50°C 45 s, 72°C 30 s, 30 cycles. The PCR results determined that: Δ Ct = Ct gene of interest - CtGAPDH [8].

2.6. Immunohistochemistry

The mice were sacrificed 30 days after treatment, and gastric cancer tissues and metastatic liver tumor tissues were taken and embedded into wax blocks, and conventional 5 μ m sections were prepared. The protein expression of HMGB1, RAGE, NF- κ B, VEGF and MT1-MMP was detected by semi-quantitative immunohistochemistry. The positive ratio in the immunohistochemical images was determined after image acquisition. 5 slices per group, at least 5 fields (x200) were analyzed for each slice.

2.7. Statistical Analysis

The experimental data were expressed as mean \pm standard deviation (\pm s). SPSS 13.0 statistical software was used. Multiple groups were compared for one-way analysis of variance. Pairwise comparison was performed for LSD-t test. P<0.05 was considered statistically significant.

3. Results

3.1. EP inhibits Orthotopic Transplantation of Gastric Cancer and Its Liver Metastasis in SCID Mice

Compared with the control group $[(883.27 \pm 96.15) \text{mm}^3, (1.12 \pm 0.28) \text{g}]$, the volume and weight of gastric cancer in the treatment group of EP 50mg/kg and EP 100mg/kg were $[(417.73 \pm 73.9) \text{mm}^3, (0.50 \pm 0.21, \text{ respectively). g}]$ and $[(309.95 \pm 53.7) \text{mm}^3, (0.31 \pm 0.16) \text{g}]$, significantly decreased, the difference was statistically significant ($P < 0.01$). There was no statistically significant difference between the EP 50mg/kg and EP 100mg/kg treatment groups ($P > 0.05$), as shown in Figure 1.

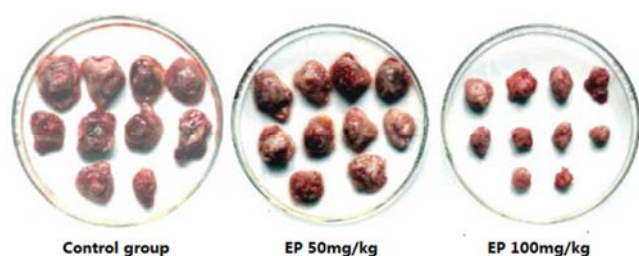


Figure 1. EP inhibits the growth of orthotopically transplanted gastric cancer tissue in SCID mice.

Compared with the control group (32.74 ± 3.81) , the number of liver tumor nodules in the EP 50mg/kg and EP 100mg/kg treatment groups were 21.55 ± 2.74 and 12.73 ± 1.64 ,

respectively, and the difference was statistically significant ($P < 0.05$). There was also a statistically significant difference between the EP 50 mg/kg and EP 100 mg/kg treatment groups ($P < 0.05$), as shown in Figure 2.

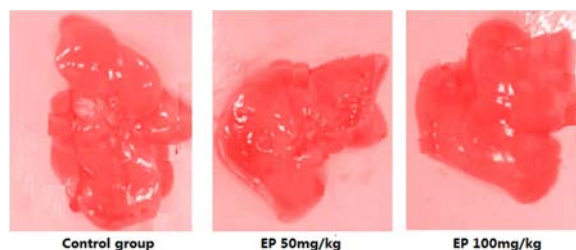


Figure 2. EP reduces the number of liver metastases in orthotopically transplanted gastric cancer in ECID mice.

3.2. Effects of EP on the Expression of HMGB1, RAGE, NF- κ B, VEGF and MT1-MMP mRNA in Orthotopically Transplanted Gastric Cancer and Metastatic Liver Tumors

The RNA in each group of gastric cancer and metastatic liver tumor tissues was extracted, and the mRNA expression levels of HMGB1, RAGE, NF- κ B, VEGF and MT1-MMP in each group were detected by real-time quantitative PCR. The results are shown in Table 1. Compared with the control group, EP inhibited the mRNA expression of HMGB1, RAGE, VEGF and MT1-MMP in gastric cancer and metastatic liver tissues, but had no significant effect on the expression of NF- κ B.

Table 1. Effect of EP on the expression of HMGB1, RAGE, NF- κ B, VEGF and MT1-MMP mRNA in orthotopically transplanted gastric cancer and metastatic liver tumor.

Group	HMGB1	RAGE	NF- κ B	VEGF	MT1-MMP
Gastric cancer					
Control group	7.12 \pm 0.64	7.94 \pm 0.54	8.11 \pm 0.65	9.22 \pm 0.57	8.53 \pm 0.48
EP 50mg/kg	8.06 \pm 0.69	9.26 \pm 0.66*	8.47 \pm 0.60	9.94 \pm 0.59	8.86 \pm 0.47
EP 100mg/kg	9.22 \pm 0.73*	9.91 \pm 0.65*	9.38 \pm 0.51	10.41 \pm 0.48*	9.93 \pm 0.64*
Metastatic liver tumor					
Control group	8.43 \pm 0.66	8.63 \pm 0.63	10.43 \pm 0.60	7.57 \pm 0.59	7.28 \pm 0.57
Group	9.86 \pm 0.74*	9.95 \pm 0.77*	11.06 \pm 0.69	9.04 \pm 0.62*	8.99 \pm 0.58*
Gastric cancer	10.24 \pm 0.77*	10.84 \pm 0.78*	11.32 \pm 0.73	9.53 \pm 0.66*	9.70 \pm 0.65*

Note: * $P < 0.05$ compared with the control group.

Effect of EP on the expression of HMGB1, RAGE, NF- κ B, VEGF and MT1-MMP protein in orthotopically transplanted gastric cancer and metastatic liver tumor

The protein expression levels of HMGB1, RAGE, NF- κ B, VEGF and MT1-MMP in each group were detected by

immunohistochemistry. The results are shown in Table 2. Compared with the control group, EP inhibited the expression of HMGB1, RAGE, VEGF and MT1-MMP in gastric cancer and metastatic liver tissue, but had no significant effect on NF- κ B expression, see Figure 3.

Table 2. Effect of EP on the expression of HMGB1, RAGE, NF- κ B, VEGF and MT1-MMP in orthotopically transplanted gastric cancer and metastatic liver tumor.

Group	HMGB1	RAGE	NF- κ B	VEGF	MT1-MMP
Gastric cancer					
Control group	973.7 \pm 163.8	1648.7 \pm 174.5	1765.8 \pm 174.9	1975.7 \pm 163.8	2062.6 \pm 153.1
EP 50mg/kg	547.5 \pm 94.6*	589.3 \pm 85.7*	1409.7 \pm 142.3	952.7 \pm 98.3*	1849.0 \pm 142.7
EP 100mg/kg	306.9 \pm 64.7*	475.7 \pm 84.9*	1297.1 \pm 98.2	582.8 \pm 63.1*	484.7 \pm 94.3*
Metastatic liver tumor					
Control group	3257.8 \pm 175.8	1286.8 \pm 97.6	3286.6 \pm 196.5	3748.5 \pm 196.3	3849.2 \pm 181.8
EP 50mg/kg	2563.8 \pm 129.7	639.6 \pm 85.9*	2957.8 \pm 164.0	3530.9 \pm 187.5	3739.1 \pm 165.2
EP 100mg/kg	1646.5 \pm 99.5*	497.9 \pm 74.1*	2677.2 \pm 158.3	964.8 \pm 107.5*	1053.7 \pm 93.7*

Note: * $P < 0.05$ compared with the control group.

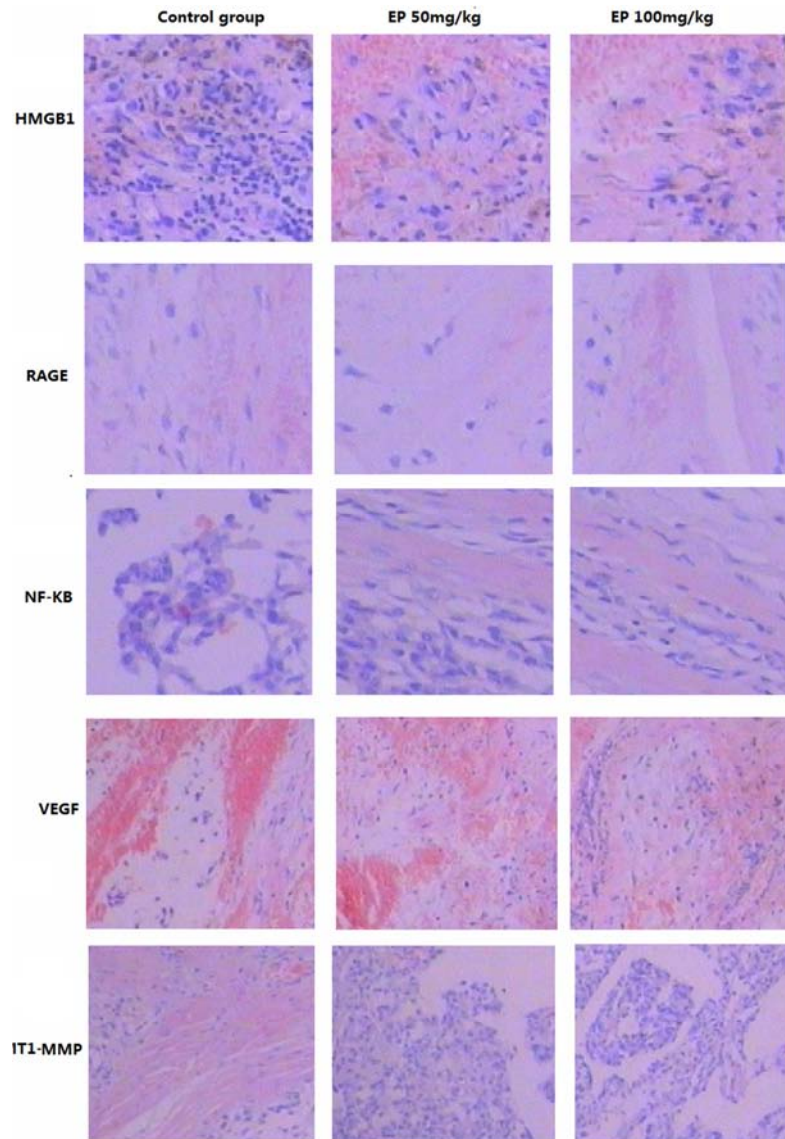


Figure 3. Immunohistochemistry showed the effect of EP on the expression of HMGB1, RAGE, NF- κ B, VEGF and MT1-MMP in orthotopically transplanted gastric cancer and metastatic liver tumor tissues ($\times 200$).

4. Discussion

Gastric cancer is a multi-gene disease. Different genes involve different pathways in the process of inducing tumorigenesis. Genetic intervention for the key targets of these pathways has become a hot spot in cancer therapy. HMGB1 is a non-histone DNA-binding protein and molecular pattern related to extracellular injury, and is also an important regulator of cell survival [9]. The biological activity of HMGB1 is mainly involved in life activities such as tumor neovascularization, apoptosis, invasion and metastasis through interaction with its corresponding receptor RAGE [10-11]. The HMGB1-RAGE pathway is also closely related to the development of gastric cancer. In the evaluation of HMGB1 overexpression and clinicopathological features of resectable gastric cancer, HMGB1 was overexpressed in almost all cancer tissues compared with non-cancerous tissues, and HMGB1 expression was negatively correlated with survival in patients with gastric

adenocarcinoma after surgical resection [12-13]. This indicates that HMGB1 plays an important role in the development of gastric cancer and may be a prognostic indicator for patients with gastric cancer after surgery or adjuvant chemotherapy. Other patients with gastric cancer found that 67% of RAGE was overexpressed, of which 92% of RAGE was overexpressed in poorly differentiated gastric adenocarcinoma, and RAGE was closely related to the depth of invasion and lymph node metastasis of gastric cancer. By detecting the expression level of HMGB1 in the serum of patients after surgery, it has certain significance for the prognosis of patients [14].

Gastric cancer is one of the common malignant tumors, ranking first in the mortality rate of malignant tumors in China. Tumor invasion and metastasis are the main causes of death in patients with gastric cancer. EP is a potent HMGB1 release inhibitor. It is a derivative of pyruvic acid with the same biological properties as pyruvic acid but more stable than pyruvate [15-16]. In endotoxin-stimulated macrophages, EP

inhibits the release of tumor necrosis factor and HMGB1 and activation of p38 mitogen-activated protein kinase and NF- κ B signaling pathways. In addition to its anti-inflammatory properties, EP also has potent anti-angiogenic activity. It inhibits vascular growth cascades by down-regulating the NF- κ B signaling pathway, including growth, invasion, migration, and tubular formation [17-18]. Therefore, EP as a multifunctional drug candidate may have potential for anti-angiogenesis and tumor therapy. Rundhaug *et al* also found that in skin cancer, EP can reduce the infiltration of NK cells, monocytes and T and B lymphocytes, and the down-regulation of interleukin-6 and HMGB1 levels, and induce the increase of apoptotic cells. [19]. Thus, by inhibiting the inflammatory effects of tumor-induced apoptosis and host, EP inhibits the growth of liver tumors, and other therapeutic approaches may play a role in the treatment of cancer.

5. Conclusion

In this study, we used SGC-7901 human gastric cancer tissue to transplant SCID mice to establish a gastric cancer liver metastasis model. The results showed that compared with the control group, the weight and size of gastric cancer tissue and the number of metastatic liver nodules were significantly higher in the EP treatment group. cut back. We know that VEGF and MT1-MMP are important molecules involved in tumor metastasis. The expression of VEGF is closely related to tumor angiogenesis, differentiation and lymph node metastasis. The expression of MT1-MMP on the surface of tumor cells activates pro-MMP to accelerate tumor metastasis and is a powerful predictor of distant metastasis of gastric cancer [20]. Inhibition of tumor cell migration and invasion by specific down-regulation of MT1-MMP expression. Our study showed that EP inhibited the expression of HMGB1, RAGE, VEGF and MT1-MMP in gastric cancer and metastatic liver tissue, but had no significant effect on NF- κ B expression. Therefore, EP may inhibit the orthotopic transplantation of gastric cancer and liver metastasis in SCID mice by down-regulating the HMGB1-RAGE pathway. EP combined with other treatments may play a greater role in the treatment of cancer, which provides new methods and strategies for the treatment of cancer.

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