

## RESEARCH COMMUNICATION

## Anticancer Effects of Huaier are Associated with Down-Regulation of P53

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## Abstract

This study was designed to explore the mechanism of Huaier anticancer effects on experimental hepatocellular cancer (HCC) development. Seventy five rats were divided into 5 groups, administered N-nitrosodiethylamine (groups B, C, D and E) or natural saline (group A). Rats in group C and E were also given Huaier. At the 15 week sacrifice point, the HCC incidence of group C was lower than group correlating with serum AFP. The serum ALT concentration (98.9% greater) and P53 mRNA levels (23.2% ) were higher in Group B than group C. Longer term survival rates between group D and E were not significantly different. Huaier can protect liver from chemical injury and furthermore HCC development, possibly with involvement of down-regulation of P53.

**Keywords:** Huaier - hepatocellular carcinoma - anticancer effect - animal model

*Asian Pacific J Cancer Prev*, 12, 2251-2254

## Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancy and is the second leading fatal disease in China (El-Serag, 2002). It is the fourth most common cause of cancer mortality and its morbidity has always been going up over the past decades both in China and across the world (Jemal et al., 2007; Llovet and Bruix, 2008; Yuen et al., 2009). Besides the routine surgical treatment, including liver transplantation, hepatectomy, local ablations and so on. Huaier, as a traditional Chinese medicine, has been proved to be equally effective on cancer treatment, such as breast cancer (Zhang et al., 2010). However, the anticancer mechanism of Huaier is still unclear and little literature has been reported on this field so far. In this study, we aim to explore the anticancer mechanisms of Huaier through treating rats with HCC.

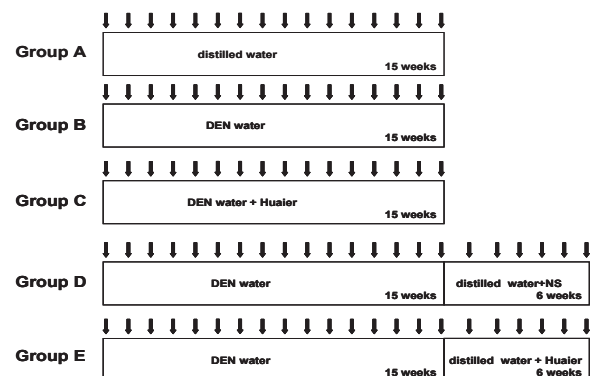
## Materials and Methods

*Animal model and experimental design*

A total of 75 male SD rats weighting 100~150g were obtained from Zhejiang Academy of Medical Sciences (Hangzhou, China). Each rat was housed under controlled temperature (20±2°C) and lighting (08:00–20:00) conditions with food and water. All rats were tested pathogen-free. All animal experiments were performed in compliance with the standards for animal use and care set by the Institutional Animal Care Committee. DEN was obtained from Sigma Co. (No. 0756, Sigma Co. USA) and Huaier was obtained from Gaitianli Pharmacy Co. (Qidong, China).

All 75 rats were randomly divided into five groups

(15 in each group, Figure 1): group A, control group, administrated via nasal stomach tube with natural saline (NS) 2 mL/rat once per day for 15 weeks continuously; group B, carcinoma induction group, DEN was given dissolved in distilled water (0.01%) for 15 weeks; group C, carcinoma induction and Huaier treated group, the same carcinoma inducing method as group B and administrated simultaneously with Huaier via nasal stomach tube, 2 mL/rat once per day (original liquid diluted for 1:1 with ddH<sub>2</sub>O), for 15 weeks continuously; group D, carcinoma induction and natural saline treated group, the same carcinoma inducing method as in group B and subsequently administrated with NS via nasal stomach tube 2 mL/rat once per day for 6 weeks continuously; group E, carcinoma induction and Huaier treated group, the same carcinoma inducing method as in group B and subsequently administrated with Huaier via nasal stomach tube, 2 mL/rat once per day (original liquid diluted for 1:1 with ddH<sub>2</sub>O), for 6 weeks continuously.



**Figure 1.** Experimental Design

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After 15-weeks administration, rats in Group A, B and C were sacrificed under anesthesia by pentobarbital. Blood samples were obtained via cardiac puncture and serum samples were stored at -30°C until analysis. Immediately after excision, livers were split into two sections. One for histological examination was stored in 10% neutral buffered formalin, the other for protein studies was stored at 80°C. What's more, rats in Group D and E were dynamically observed for survival investigation.

### Histology

For histologic analysis, liver sections (4mm) were stained with hematoxylin and eosin (H&E) according to standard procedures and were analyzed by an expert pathologist. Neoplastic nodules and HCC were diagnosed on the basis of the published criteria (Squire RA and Levitt MH, 1975).

### Biochemical parameters

The tissue and serum Alpha Fetal Protein (AFP) were measured by a rat alpha-fetoprotein ELISA kits (R&D Systems, Minneapolis, MN USA). Serum alanine aminotransferase (ALT) was estimated by an autoanalyser using regular commercial kits.

### RNA isolation, reverse transcription and real-time polymerase chain reaction of P53

The mRNA levels of P53 were analyzed by real-time polymerase chain reaction (real-time PCR). Total RNA was collected from the frozen liver tissue using TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA). Reverse transcription and quantitative polymerase chain reaction analysis was carried out as previously described; data were normalized to the expression of the P53 gene. The following primer were used: P53 forward: 5'-ATG CTG AGT A TC TGG ACG ACA-3', P53 reverse: 5'-CAGGC ACAA CACGA ACC-3'.

### Follow up of the Rats

After 15-weeks chemical induction, rats in Group D were administrated with NS via nasal stomach tube, 2 mL/rat once per day for 6 weeks continuously and rats in Group E were administrated with Huaier via nasal stomach tube, 2 mL/rat once per day for 6 weeks.

### Statistical analysis

Quantitative variables were expressed as mean  $\pm$  SD. Student's t test was used to compare quantitative variables. All the variables were detected by univariate analysis and a P value of less than 0.05 was considered statistically significant. SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL) was used to complete all the analyses.

## Results

Twenty rats died during the experiment (7 in group B, 4 in group C, 5 in group D and 4 in group E). The main reasons for death were oesophagitis and/or thorax and celiac infection caused by perforation because of pouring reagent into the stomach. The inter-group comparison of body weights showed there was significant difference

**Table 1. Animal Body Weight (in Grams), Liver Weight (in Grams), Ratio of Liver-to-animal Weight (in %) in 3 Groups**

	Group A (n=15)	Group B (n=8)	Group C (n=11)
Animal body(g)	421.7 $\pm$ 3.41	188.9 $\pm$ 12.6*	186.8 $\pm$ 25.6*
Liver (g)	13.65 $\pm$ 0.15	14.5 $\pm$ 1.32	13.4 $\pm$ 2.05
Liver to rat ratio	3.24 $\pm$ 0.06	7.87 $\pm$ 1.01*	7.13 $\pm$ 0.25*

\*P<0.01 Group A v.s Group B; Group A v.s Group C

**Table 2. The Comparison of Carcinoma in Group B and Group C**

	Group B (n=8)	Group C (n=11)
Incidence of HCC	100% (8/8)	54.5% (6/11)
Total tumor numbers	16	8*
Average tumor numbers in one rat with HCC	2.00	1.33
Total tumor Diameter (cm)	10.7	7.80
Average tumor Diameter (cm)	0.67 $\pm$ 0.31	0.98 $\pm$ 0.37

\*P<0.05, Group B vs Group C

**Table 3. The Concentration of AFP in Serum and Tissue (ng/ml)**

	Serum	Tissue		
		HC tissue	Adjacent tissues	Cirrhosis tissue
Group A	19.0 $\pm$ 1.74			
Group B	40.0 $\pm$ 3.47*	68.5 $\pm$ 1.84	69.17 $\pm$ 12.9	73.6 $\pm$ 7.61
Group C	28.0 $\pm$ 2.52*	60.1 $\pm$ 5.23	52.4 $\pm$ 3.60	71.29 $\pm$ 7.54

\*P<0.05 Group B v.s Group A; Group C v.s Group A; R&D, Corporation Enzyme Linked Immunosorbent Assay (ELISA): Rat Alpha-fetoprotein

**Table 4. Liver Function of the Rats in 3 Groups**

	Group A	Group B	Group C
ALT	65.67 $\pm$ 5.24	221.20 $\pm$ 14.20*	155.67 $\pm$ 12.14 <sup>#</sup>

\*P<0.01 Group B v.s Group A; <sup>#</sup>P<0.05 Group C v.s Group B

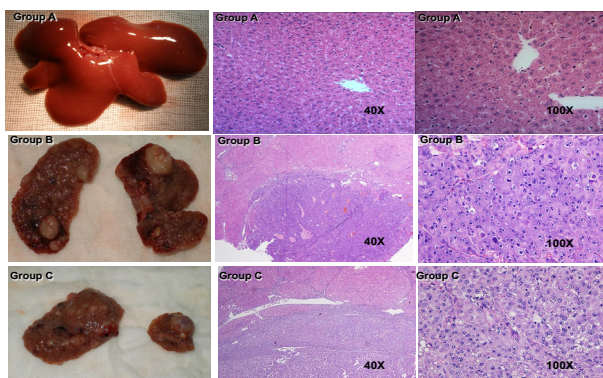
between group A and group B, group A and group C on animal body weight and liver to rat weight ratio (P<0.001). The liver weights and liver to rat weight ratio between group B and group C were not significantly different (P>0.05). Neither were liver weights among the 3 groups (P>0.05).

### Carcinoma development

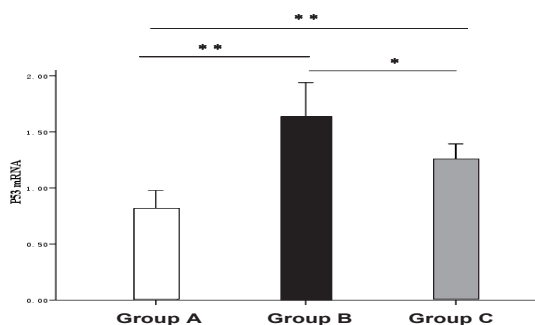
There incidence of HCC in group C is much lower than that in group B (54.5% vs 100, P<0.05). More details on HCC between the two groups are shown in Table 2. The macroscopic and histological appearance of the liver is showed in Figure 2.

### The comparison of AFP concentration and serum ALT concentration between different groups

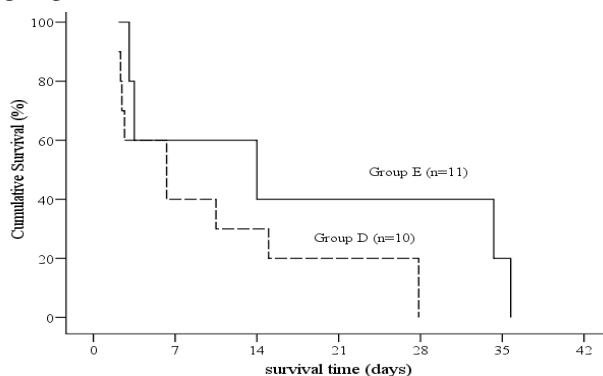
There was significant difference between group A and B, group A and C on serum AFP concentration (P<0.05). No significant differences were found between group B and group C on tissue AFP concentration (P>0.05). There was significant difference between group A and B on serum ALT concentration (P<0.01), group C and B (P<0.05). Results were summarized in Tables 3 and 4.



**Figure 2. Macroscopic and Histological Appearance of Typical Livers and Lesions**



**Figure 3. mRNA Expression of P53 in the 3 Groups.** Data are mean + S.D.\*P < 0.05 for group C vs. group B; \*\*P < 0.01 for group B vs. group A and for group C vs. group A



**Figure 4. The Comparison of Cumulative Rat Survival Between Group D and E (P>0.05)**

*The comparison of mRNA levels of P53*

The mRNA level of p53 significantly greater in group B compared to group A (P < 0.01; Figure 3.). In Huaier-treated animals, liver P53 mRNA decreased compared with DEN-treated animals (P < 0.05).

*Survival of rats*

The survival curve was shown in Figure 4. There were no significant difference of rats survival rates (2-week and 3-week) between Group D and E ( 30% vs 40% and 20% vs 40%, P > 0.05 for both).

**Discussion**

It is well known that DEN can lead to impairment of the nuclear enzymes involved in DNA repair/replication and has been widely used as a carcinogen to induce

HCC animal models (Jagan et al., 2008). It has been confirmed that oxidative stress plays an important role in carcinogenesis and reactive oxygen species (ROS) are important contributions to both tissue injury and DNA damage (Beckman and Ames, 1997; Ramakrishna et al., 2006; Marengo et al., 2010). After metabolism, DEN is transformed to reactive electrophilic reactants which could alter the structure of DNA and form alkyl DNA adducts, inducing chromosomal aberrations and micronuclei in the rat liver (Yoshiji et al., 1991).

The treatment of HCC is still a huge challenge in the world. There is an urgent need for effective therapy to prevent and treat HCC. In this study, we evaluated whether Huaier, as a Traditional Chinese Medicine, was a good alternative for HCC prevention on DEN-induced model. In this study, we found that the HCC incidence of Huaier treatment rats is much lower. When administrated along with DEN induction, Huaier can effectively prevent the development of HCC in rats.

As one of the most sensitive intracellular enzymes, ALT is an key indicator for liver function evaluation (Ozturk et al., 2006; Ramakrishnan et al., 2007; Liang et al., 2009; Miyake et al., 2009). Our present study showed DEN toxication lead to a significant increase in serum ALT level due to excessive production from normal hepatocytes and HCC cells. Ramakrishnan et al. reported that DEN toxication could increase the permeability of cell membrane resulting in release of ALT into serum (Ramakrishnan G et al., 2007). Huaier treatment significantly attenuated the increased activity of ALT due to its promoting effects on hepatocyte regeneration, and membrane protection which can decrease enzyme leakage (Jadon A et al., 2007).

AFP is synthesized by fetal cells and can be detected in serum at levels of 20 ng/ml in 75.8%-78.8% of HCC patients in China (Zheng et al., 2005). It is used as the most valuable serum tumor marker for early diagnosis of HCC and can monitor tumor recurrence after hepatectomy (Cha et al., 2003; Ren et al., 2006; Xu et al., 2009). However, the AFP in 20%-25% of patients with chronic hepatitis and liver cirrhosis can also rise up to 2500 mg/L (Libbrecht et al., 2006 Zinkin et al., 2008). The current study showed the AFP in HCC model group are higher than that in control group but there is no significant difference on AFP level after Huaier treatment, which reveals that maybe the high level of AFP in this study is not associated with HCC but chronic hepatitis and liver cirrhosis.

As a tumor suppressor gene, P53 was found mutated in approximately 50% of human tumors and plays an important role in the response to genotoxic stress and hypoxia (Vogelstein et al., 2000). Under normal conditions, p53 is a short-lived protein that is present in cells at a barely detectable level. Exposure to various exogenous stress including DNA damage, hypoxia, heat shock, etc., P53 could be activated resulting in DNA repair and apoptosis of impaired cells (Chipuk and Green, 2006; Boisvert and Lamond, 2010; Momota et al., 2010). In this study, p53 mRNA increased significantly in the DEN group. Over-expression of p53 was related to the cellular stress induced by DEN, reflected by the inflammatory/fibrotic environment, the presence of cell

necrosis and proliferative hepatocytes, while the p53 mRNA decreased in Huaier-treatment rats. It is possibly due to the anti-inflammation and fibrosis effects of Huaier, which led to less reactive p53 activation. In addition, we found Huaier didn't improve the survival rate of rats with HCC. This may indicate that there is no value with Huaier administration if HCC had generated.

In summary, application of Huaier showed a suppressing effect on HCC development and it also had protective effect on chemical liver injury. The anticancer effect of Huaier is associated with the down-regulation of P53.

## Acknowledgements

This work was supported by the National Basic Research Program of China (973 program 2009CB522404), the National Key Technology R&D Program (2008BA160B03) and the key project of Administration of Traditional Chinese Medicine of Zhejiang Province (No. 2005Z007).

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