

## Effects of Tea on Preneoplastic Lesions and Cell Cycle Regulators in Rat Liver

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### Abstract

The effects of tea polyphenols and tea pigments on rat liver precancerous lesions and some cell cycle regulators were studied. A modified Solt-Farber model in rats was established by multiple low-dosage of *N*-nitrosodiethylamine (NDEA) i.p. injections, followed by i.p. CCl<sub>4</sub> injection and partial hepatectomy. Sixty male Wistar rats were randomly divided into four groups: positive control group, two tea-treated groups, and negative control group. Rats in tea-treated groups were given tea polyphenols (0.1%) and tea pigments (0.1%) in drinking fluid during the whole experiment. The number and area of glutathione S-transferase P (GST-P)-positive foci in the rat liver were used as biomarkers of precancerous liver lesions. Western blotting assay was carried out to detect the expression of cyclin D1, CDK4, and P21<sup>WAF1/CIP1</sup> on whole liver extract. At the end of the experiment (56 days), the number and area of GST-P-positive foci in liver increased significantly in carcinogen-administered positive control group, whereas no GST-P-positive foci were found in the negative control group in which animals did not receive carcinogen exposure. The number and area of GST-P-positive foci in tea-treated, carcinogen-exposed groups were significantly reduced as compared with the positive control group. It was also found that the expression of P21<sup>WAF1/CIP1</sup> was significantly induced and the expression of cyclin D1 and CDK4 was significantly inhibited in tea-treated groups. These results suggest that tea polyphenols and tea pigments are effective in preventing the precancerous liver lesions in rats, and modulation of cell cycle by regulating cell cycle regulators may be a possible mechanism.

### Introduction

Tea is one of the most popular beverages consumed in the world. The two major types of tea consumed are black tea and green tea. Green tea is predominant in China, Japan, and a few countries in North Africa and the Middle East, and it is rich in

polyphenols such as EGCG<sup>2</sup>, (–)-epicatechin, (–)-epicatechin gallate, and epigallocatechin. Black tea is consumed mainly in western countries. The main ingredients of black tea is tea pigments such as theaflavins and thearubigins. The anticarcinogenic and antimutagenic properties of tea were first demonstrated a decade ago (1, 2). Since then, tea polyphenols and tea extract have been shown in a number of animal models to prevent against chemically-induced carcinogenesis in lung, forestomach, esophagus, duodenum, pancreas, breast, colon, and liver (3–5). Although not conclusive, epidemiology studies have suggested a protective effect of tea consumption against human cancers (6, 7).

Altered hepatocellular foci, a specific enzymatic phenotype, is considered a preneoplastic lesion during chemically-induced rat hepatocarcinogenesis (8). Immunohistochemical identification of GST-P was the most widely used method for detection (9, 10). GST-P-positive foci was recognized a reliable and sensitive marker, and it has been widely used in the short term or midterm assay for carcinogen screening and chemoprevention studies on cancer (11, 12).

The eukaryotic cell cycle is regulated by signal transduction pathways mediated by a series of cell cycle regulators. Cyclins are positive regulators of cell cycle progression. They function by forming a complex with and activating CDKs. CDK inhibitors are negative regulators of the cell cycle. They bind to and inhibit the activity of cyclin-CDK complexes (13–15).

Yamane *et al.* (16) demonstrated that 0.01% and 0.1% green tea polyphenols inhibited the development of azoxymethane-induced colon carcinogenesis. Tamura *et al.* (17) also found that 0.01% and 0.1% green tea extract decreased the number of GST-P-positive foci. Therefore, in the present study, we used a modified Solt-Farber model to examine the effect of 0.1% tea polyphenols and 0.1% tea pigments on GST-P-positive foci; and at the same time, to explore related mechanisms.

### Materials and Methods

**Materials.** Tea polyphenols and tea pigments were provided by the Institute of Tea Science and Research, Chinese Academy of Agricultural Sciences (Hangzhou, China). The purity of tea polyphenols was 40%, and it is composed mainly of polyphenolic compounds: EGCG (13%), (–)-epicatechin (3%), (–)-epicatechin gallate (6%), and epigallocatechin (6%). The residue of these tea polyphenols includes caffeine, sugars, amino acids, and moisture. Tea pigments are the oxidative products of 40% tea polyphenols, which are primarily composed of theaflavins (2%) and thearubigins. NDEA was obtained from Sigma Chemical Co. The rabbit anti-rat GST-P polyclonal antibody

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<sup>2</sup>The abbreviations used are: EGCG, (–)-epicatechin; GST-P, glutathione S-transferase P-form; CDK, cyclin-dependent kinase; NDEA, *N*-nitrosodiethylamine.

Table 1 Food intake in each group<sup>a</sup>

Group	No. of animals	Weeks 1–2	Weeks 3–4	Weeks 5–6	Weeks 7–8
Positive control	15	130.6 ± 22.9	147.1 ± 12.3	152.9 ± 11.7	141.6 ± 21.0
Tea polyphenols	15	119.2 ± 20.7	140.3 ± 19.2	142.3 ± 16.2	134.7 ± 21.6
Tea pigments	15	117.5 ± 13.7	139.5 ± 12.4	149.5 ± 12.3	131.8 ± 24.0
Negative control	15	131.7 ± 13.5	149.0 ± 12.5	155.3 ± 13.3	148.2 ± 18.7

<sup>a</sup> Values are means ± SD.

was a gift from Dr. Kimihiko Satoh of Hirosaki University School of Medicine in Japan.

**Animals and Treatment.** Sixty male Wistar rats, 70–90 g body weight, were purchased from the Animal Center of the Chinese Academy of Medical Sciences (Beijing, China) and fed regular chow (no aflatoxins contamination). Rats were randomly divided into four groups, 15 rats in each group. The animals were maintained in a controlled environment at 24 ± 1°C and 50 ± 10% relative humidity with the altering 12:12-hour light-dark cycle. A modified version of the Solt-Farber method (18) was used to generate the liver GST-P-positive foci that indicate precancerous liver lesion. The positive control group was treated as: NDEA (10 mg/kg body weight, i.p.) was given once a day from days 7–12, and 20% CCl<sub>4</sub> (0.5 ml/rat, i.p.) was given once a day on days 40 and 41, and finally the rats were subjected to two-thirds hepatectomy under ether anesthesia on day 42. Besides these treatments, the tea-treated groups were treated with 0.1% tea polyphenols or 0.1% tea pigments as drinking fluid during the whole experiment. Rats in the negative control group were injected i.p. with the equal volumes of saline instead of carcinogen. Rats in the positive control group and the negative control group were given tap water as drinking fluid. All the rats were sacrificed on day 56, and the livers were cut into several sections for GST-P immunohistochemical staining and Western blot assays.

**GST-P Immunohistochemical Assay.** Immunohistochemical assay was performed using the avidin-biotin peroxidase complex immunoperoxidase methods (19). Three different sections from each liver were fixed with 10% buffered formalin and then embedded in paraffin blocks. The blocks were cut into 4-μm serial slices and mounted on glass slides. The deparaffinized sections were then incubated with methanol containing 3% H<sub>2</sub>O<sub>2</sub> for 10 min to block the activity of endogenous peroxidases. Then, the slices were treated with normal sheep serum to reduce nonspecific reactions and incubated at 4°C overnight with rabbit antirat GST-P polyclonal antibody and then biotinylated antirabbit secondary antibody and avidin-biotin peroxidase complex for 30 min consecutively. The reactivity was carried out with 0.03% diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. The slices without primary antibody treatment were used as the negative control. A focus with more than 10 positive cells was considered a positive focus (20). The area and number of GST-P-positive foci were analyzed by the Image Pro-Plus image system.

**Determination of P21<sup>WAF1/CIP1</sup>, Cyclin D1, and CDK4 (Western blot).** Six rats in each group were randomly chosen for the determination of P21<sup>WAF1/CIP1</sup>, cyclin D1, and CDK4 protein expression by Western blot assay. Rat liver tissue was homogenized in a Polytron with four volumes of PBS, and the homogenate was centrifuged at 9,000 × g for 15 min and then at 105,000 × g for 20 min. The amount of protein in the supernatant was quantified using the method of Lowry *et al.* (21). Thirty micrograms of protein were subjected to 10% SDS-polyacrylamide gels to separate the protein according to

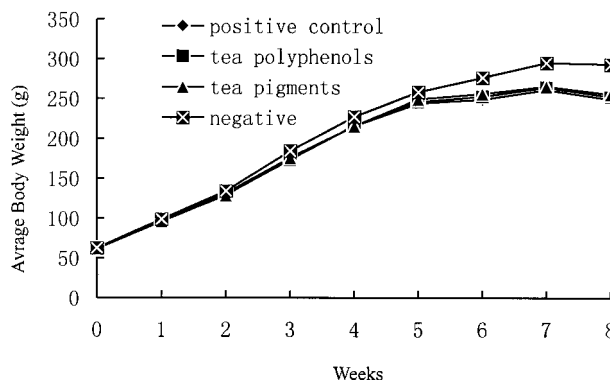


Fig. 1. Effects of tea treatment on body weight of rats. Body weight was measured once a week. No differences were observed before partial hepatectomy among the four groups. At the eighth week, body weight in the NDEA-treated groups was significantly lower than that of the negative control group ( $P < 0.05$ ).

the method of Laemmli (22). The separated protein was transferred onto nitrocellulose membranes, and each membrane was cut into two pieces; one piece was incubated at 4°C overnight with polyclonal primary antibody P21<sup>WAF1/CIP1</sup>, cyclin D1, or CDK4, and the other one with β-actin (used as a control for protein loading). All antibodies were obtained from Zymed Laboratories (San Francisco, CA). Then membranes were incubated at 37°C for 1 h with secondary antibody conjugated with peroxidase, and the signal was detected using the chemiluminescent detection luminol (Santa Cruz Biotechnology, Santa Cruz, CA). Relative protein level was calculated as the ratio of the optical density of P21<sup>WAF1/CIP1</sup>, cyclin D1, or CDK4 and that of β-actin.

**Statistical Analysis.** Statistical analysis of data was performed using the Student's *t* test. To obtain a normal distribution, immunohistochemical data were analyzed after square root transformation.

## Results

**Food Intake, Body Weight, and Tea or Water Consumption.** There were no significant differences in food intake of each group (Table 1). The body weight growth of rats in each group during the experimental period also showed no differences before partial hepatectomy (Fig. 1). At the eighth week, body weight in NDEA-treated groups was significantly lower as compared with the negative control group ( $P < 0.05$ ). The amount of tea or water drinking among different groups during the whole experimental period showed no significant differences (Table 2).

**Effect of Tea Polyphenols on GST-P-positive Foci.** The results of GST-P immunohistochemical staining are shown in Table 3. All animals in the NDEA-treated groups developed positive foci in liver slices, whereas no positive foci were found

Table 2 Water or tea consumption (milliliters)<sup>a</sup>

Group	No. of animals	Weeks 1–2	Weeks 3–4	Weeks 5–6	Weeks 7–8
Positive control	15	21.9 ± 3.30	27.2 ± 2.37	27.5 ± 2.27	29.1 ± 2.28
Tea polyphenols	15	22.0 ± 3.67	26.0 ± 2.09	26.9 ± 2.44	29.2 ± 2.43
Tea pigments	15	24.2 ± 4.04	27.9 ± 3.36	28.5 ± 2.10	29.2 ± 1.56
Negative control	15	23.5 ± 2.87	27.0 ± 2.50	28.5 ± 2.91	29.4 ± 2.31

<sup>a</sup> Values are means ± SD.

Table 3 Effects of tea on rat liver GST-P-positive foci

Group	n	GST-P-positive foci	
		Foci/cm <sup>2</sup>	Area (mm <sup>2</sup> /cm <sup>2</sup> )
Positive control	10	5.70 ± 2.78	3.72 ± 2.39
Tea polyphenols	10	3.10 ± 1.58 <sup>a</sup>	1.37 ± 0.77 <sup>b</sup>
Tea pigments	10	2.70 ± 2.10 <sup>a</sup>	1.49 ± 1.00 <sup>b</sup>
Negative control	10	0	0

<sup>a</sup> Statistically different from the positive control group,  $P < 0.01$ .

<sup>b</sup> Data were analyzed after square root transformation, statistically different from the positive control group,  $P < 0.01$ .

in the negative control group. The number of GST-P-positive foci was significantly reduced in rats drinking 0.1% tea polyphenols and tea pigments as compared with the positive control group, and the average area of GST-P-positive foci was also significantly reduced in the tea-treated groups.

**Effect of Tea Polyphenols on P21<sup>WAF1/CIP1</sup>, Cyclin D1, and CDK4 Protein Expression.** The protein expression level of P21<sup>WAF1/CIP1</sup>, cyclin D1, and CDK4 in rat liver cytosol fraction was measured by the Western blot assay (Table 4; Fig. 2). Compared with the positive control group, P21<sup>WAF1/CIP1</sup> protein relative level in rats of tea-treated groups was significantly increased, whereas the relative level of cyclin D1 and CDK4 protein was significantly reduced in tea-treated groups.

## Discussion

The present study examined the effects of tea polyphenols and tea pigments on the development of liver preneoplastic lesions GST-P and some cell cycle regulators in a modified Solt-Farber model, in which GST-P foci can be formed within 8 weeks. Because this process involves multistages of carcinogenesis, it is considered a valuable animal model for chemoprevention study. In this model, the carcinogen-initiated cells proliferate selectively, simulated by hepatectomy and rapid formation of enzyme-altered foci—GST-P-positive foci (23). In general, these foci are rarely detected in normal rat liver. Thus, GST-P-positive foci are recognized as a reliable and sensitive marker of liver carcinogenesis, and it has been widely used in carcinogen screening and chemoprevention studies for liver cancer (11). In this study, we counted the number of GST-P foci under a light microscope, and, at the same time, we measured the foci area using the Image Pro-Plus image system. We found that the number and area of GST-P-positive foci were greatly increased in the positive control group, whereas the number and area of GST-P-positive foci were significantly decreased in rats treated with tea polyphenols and tea pigments. Matsumoto *et al.* (24) found that tea polyphenols reduced not only the numbers, but also the size of the GST-P-positive foci in their study. Tamura *et al.* (17) also found that green tea extract reduced the numbers of the foci. These results are in accordance with our present study.

In the study presented here, cyclin D1, CDK4, and

P21<sup>WAF1/CIP1</sup> protein levels were measured by Western blotting analysis in normal rat liver tissues and preneoplastic lesions. In normal adult animals, hepatocytes are highly differentiated and can remain for a long period in a quiescent G<sub>0</sub> state (25). However, hepatocytes have the potential to proliferate after chemical or physical partial hepatectomy (26). Cyclins-CDKs complexes are essential for cell cycle transition. Because the major regulatory events leading to cell proliferation occur in the G<sub>1</sub> phase of the cell cycle, altered expression of G<sub>1</sub> cyclins and their CDKs may be an important step in oncogenesis (27–30). Studies in human cancer have shown that G<sub>1</sub> cyclins and their CDKs are the most consistently altered cell cycle regulatory proteins, and an accumulating body of evidence suggests that deregulated expression of cyclin D1 and CDK4 are associated with malignancy (31). Therefore, inhibition of the expression of cyclin D1 and CDK4 protein may be one way of chemopreventive agents to block the development of carcinogenesis. It has been reported that most cell cycle regulatory proteins, including cyclin D1 and CDK4, are already present in regenerating rat liver cells and related to transcriptional and translational expression (32). In this study, we observed an increase of cyclin D1 and CDK4 protein expression in liver preneoplastic lesions when compared with normal liver tissue, and tea polyphenols and tea pigments treatment significantly inhibited the expression of cyclin D1 and CDK4 proteins as compared with the positive control group. Ahmad *et al.* (33) studied the cell cycle dysregulation by green tea polyphenol and found that the major green tea polyphenol EGCG inhibited cyclin D1 and CDK4 protein expression in human epidermoid carcinoma (A431) cells. Our present animal study result confirmed this *in vitro* result.

P21<sup>WAF1/CIP1</sup>, one of the CDK inhibitors, selectively inhibits the G<sub>1</sub>/S cyclin-CDK complexes (34). Because cyclin D1/CDK4 complexes are thought to play a major role in cell cycle progression in early phase, induction of P21<sup>WAF1/CIP1</sup> and consequent inhibition of cyclin D1 and CDK4 may be an important step in the chemoprevention of cancer. Lin *et al.* (35) demonstrated that EGCG induced the expression of P21<sup>WAF1/CIP1</sup> protein, meanwhile inhibiting the expression of CDK4. Another study also showed that EGCG induced the expression of P21<sup>WAF1/CIP1</sup> and inhibited the expression of cyclin D1 and CDK4 (33). However, all these studies are *in vitro* studies; *in vivo* studies are needed to confirm these results. In the present animal study, we observed the expression of P21<sup>WAF1/CIP1</sup> protein was significantly induced by tea as compared with the positive control group. All these studies suggested that regulation of cell cycle regulators may be a possible mechanism of cancer chemoprevention by tea.

In conclusion, our study showed that tea polyphenols and tea pigments significantly reduced the number and area of GST-P-positive foci. Western blot analysis showed that tea induced the expression of P21<sup>WAF1/CIP1</sup> and inhibited the expression of cyclin D and CDK4. However, because the whole liver was used in Western blot analysis, the expression of the

Table 4 Effect of tea on cell cycle regulatory proteins level in rat liver with precancerous lesions

Group	n	Relative protein level <sup>a</sup>		
		P21 <sup>WAF1/CIP1</sup>	cyclin D1	CDK4
Positive control	6	1.87 ± 0.27	2.50 ± 0.76	2.32 ± 0.41
Tea polyphenols	6	2.76 ± 0.43 <sup>b</sup>	1.67 ± 0.50 <sup>c</sup>	1.75 ± 0.34 <sup>c</sup>
Tea pigments	6	2.66 ± 0.36 <sup>b</sup>	1.61 ± 0.38 <sup>c</sup>	1.62 ± 0.25 <sup>c</sup>
Negative control	6	1.07 ± 0.36	0.97 ± 0.42	1.15 ± 0.28

<sup>a</sup> Relative protein level calculated as the ratio of the optical density of P21<sup>WAF1/CIP1</sup>, cyclin D1, or CDK4 and that of  $\beta$ -actin.

<sup>b</sup> Statistically different from the positive control group,  $p < 0.01$ .

<sup>c</sup> Statistically different from the positive control group,  $p < 0.05$ .

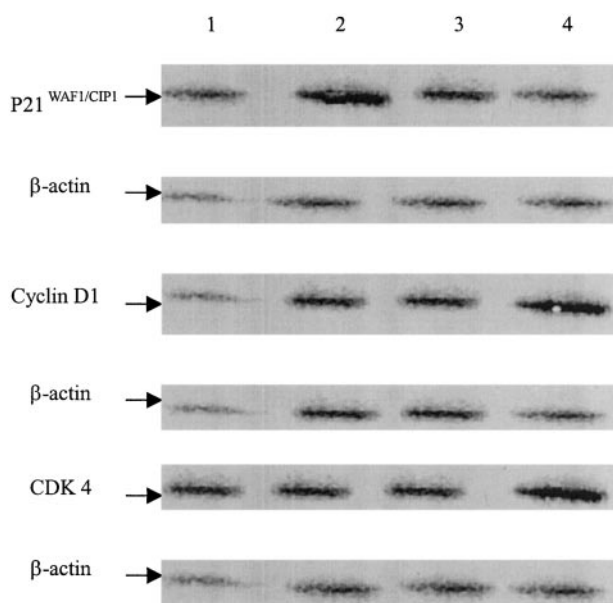


Fig. 2. Western blot analysis of cell cycle regulatory proteins. Six rats in each group were used for P21<sup>WAF1/CIP1</sup>, cyclin D1, and CDK4 protein detection. The proteins were subjected to SDS-PAGE and then to Western blotting. Membranes are probed with antibodies to P21<sup>WAF1/CIP1</sup>, cyclin D1, or CDK4, secondary antibody conjugated with peroxidase, and the signal was detected using the chemiluminescent detection system. Densitometric analysis of each band was performed to quantify the relative level of protein.  $\beta$ -Actin was used as a control for protein loading. The representative blot is shown with each lane representing analysis of sample from one rat. Lane 1, negative control; lane 2, tea polyphenols; lane 3, tea pigments; lane 4, positive control.

proteins could have come from a specific expression in the GST-P foci or a combination of expression in both the neoplastic cells as well as normal cells. Therefore, it is necessary to do immunohistochemical staining for these proteins to justify the results of the present study. Experiments on this respect will be conducted in our laboratory. Although the mechanisms of this inhibitory effect have not been fully elucidated, our study showed that modulation of cell cycle by regulating cell cycle regulators may be a possible mechanism.

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