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Phase IIa chemoprevention trial of green tea polyphenols in high-risk individuals of liver cancer: modulation of urinary excretion of green tea polyphenols and 8-hydroxydeoxyguanosine

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Modulation of urinary excretion of green tea polyphenols (GTPs) and oxidative DNA damage biomarker, 8-hydroxydeoxyguanosine (8-OHdG), were assessed in urine samples collected from a randomized, double-blinded and placebo-controlled phase IIa chemoprevention trial with GTP in 124 individuals. These individuals were sero-positive for both HBsAg and aflatoxin–albumin adducts, and took GTP capsules daily at doses of 500 mg, 1000 mg or a placebo for 3 months. Twenty-four hour urine samples were collected before the intervention and at the first and third month of the study. Urinary excretion of 8-OHdG and GTP components was measured by HPLC-CoulArray electrochemical detection. The baseline levels of 8-OHdG and GTP components among the three groups showed homogeneity ($P > 0.70$), and a non-significant fluctuation was observed in the placebo group over the 3 months ($P > 0.30$). In GTP-treated groups, epigallocatechin (EGC) and epicatechin (EC) levels displayed significant and dose-dependent increases in both the 500 mg group and 1000 mg group ($P < 0.05$). The 8-OHdG levels did not differ between the three groups at the 1 month collection, with medians of 1.83, 2.08 and 1.86 ng/mg-creatinine for placebo, 500 and 1000 mg group, respectively ($P = 0.999$). At the end of the 3 months' intervention, 8-OHdG levels decreased significantly in both GTP-treated groups, with medians of 2.02, 1.03 and 1.15 ng/mg-creatinine for placebo, 500 mg and 1000 mg group, respectively ($P = 0.007$). These results suggest that urinary excretions of EGC and EC can serve as practical biomarkers for green tea consumption in human populations. The results also suggest that chemoprevention with GTP is effective in diminishing oxidative DNA damage.

Introduction

Primary liver cancer, mainly hepatocellular carcinoma (HCC), is one of the most common cancers in Asia, Africa, and in populations of Asian- and Hispanic-Americans (1,2). Because of its poor prognosis, HCC has a mortality approaching its morbidity, and is the third cause of cancer deaths in the world (3,4). Eighty percent of the world's HCC cases arise in the developing world in Southeast Asia and sub-Saharan Africa (5), where the major etiologic factors have been identified as hepatitis B virus (HBV) infection and dietary aflatoxin (AF) exposure (6,7), with synergistic effects suggested by several epidemiological studies (8,9). Primary prevention strategies, such as HBV vaccination in infants and AF control in food, can diminish the exposure to major risk factors and have offered the best hope for reducing HCC morbidity in these communities; however, outcomes may require decades to appear. Currently, the great challenge in HCC prevention and control is how to reduce the risk in individuals who have already been exposed to both etiological risk factors for decades. Chemoprevention has been proposed as a promising strategy to help these high-risk individuals.

Green tea polyphenols (GTPs) have been shown safe and effective as chemopreventive agents in various *in vitro* bioassays and *in vivo* animal models for inhibition of carcinogen-induced mutagenesis and tumorigenesis at several target organ sites, including AFB_1-induced liver tumors (10–12). GTP is the secondary metabolite in tea plants, and accounts for 30–36% weight of the water extractable materials in tea leaves. The major GTP components include (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC), with EGCG being the most abundant (11). Though the safety and efficacy of GTP are consistent in most animal studies, human epidemiological studies have so far generated controversial results (13). Some studies found no association or even worse, a positive association between tea drinking and cancer risk, while others revealed a reduced risk of cancer in the esophagus, stomach, lung and liver with green tea consumption (14–18).

Several mechanisms have been proposed for the anti-carcinogenic effect of GTP, with the well-accepted one that GTP can capture and detoxify reactive oxygen species (ROS) produced in the process of carcinogen metabolism, inflammation, aerobic respiration and exposure to background radiations (19). ROS attack all macromolecules including lipids, proteins and DNA. The addition of hydroxyl radical (OH) to the C8-position of guanine produces C8–OH adduct radical (20), which is subsequently converted to 8-OH-guanine (8-OH-Gua) by a one-electron oxidation (21). While damaged lipids and proteins can be removed by metabolic turnover of molecules, impaired DNA has to be repaired *in situ*, or destroyed by apoptotic processes, if not to result in mutations. In humans, the 8-OH-Gua glycosylase (OGG1) is the primary enzyme for the repair of 8-OH-guanine adduct in a

Abbreviations: AF, aflatoxin; EC, epicatechin; EGC, epigallocatechin; GTP, green tea polyphenols; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; 8-OHdG, 8-hydroxydeoxyguanosine; ROS, reactive oxygen species.

short-patch base-excision repair (BER) (22). The excised form of 8-OH-Gua is 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is excreted into urine without further metabolism and is stable for a significant time (23,24). Thus, urinary 8-OHdG generally reflects the whole body's oxidative DNA damage and repair, and becomes a putative biomarker for oxidative stress (24). Detection of urinary 8-OHdG provides a sensitive and non-invasive means to evaluate the efficacy of chemoprevention.

Lack of objective data on the amount of tea consumption has hindered the precise evaluation of the association between tea ingestion and human cancer risk in questionnaire-based epidemiological studies (25–28). It has been proposed that the quantitative measurement of GTP components in human body fluids is a more appropriate way to reflect green tea consumption in prospective epidemiological studies (18,29). However, the best GTP component to serve as an exposure biomarker has not yet been determined and/or validated at the population level, and a precise evaluation of the role of GTP in cancer risk will most likely come from a prospective human intervention study.

Our work presented here is based on a randomized, double-blinded, and placebo-controlled phase IIa chemoprevention trial with GTP in a HCC high-risk population (30). The urine samples collected from this trial were analyzed for GTP components to validate biomarkers for tea consumption, and urinary 8-OHdG levels were assessed as a surrogate endpoint to evaluate the efficacy of GTP intervention in these high-risk individuals.

Materials and methods

Materials

GTP was obtained from the US–China joint venture Shili Natural Product Company, (Guilin, Guangxi) with purity >98.5%, and encapsulated by the Guangxi Pharmaceutical Company (Nanning, Guangxi). Authentic standard GTP components and 8-OHdG, and β-glucuronidase, sulfatase, ascorbic acid and creatinine detection kit were purchased from Sigma–Aldrich Chemical Company (St Louis, MO). Oasis® HLB cartridges were products of Waters Corporation (Milford, MA). All organic solvents used were of high-performance liquid chromatography (HPLC) grade. Other chemicals and reagents were purchased at the commercially highest degree of purity available.

Study design and procedure

The design, clinical outcome and baseline biomarker data of this phase IIa GTP chemoprevention trial has been previously described (30). The overall study design is shown in Figure 1. Briefly, 1200 blood samples were screened and 124 voluntary residents were enrolled into this trial. The recruiting criteria include healthy adults with positive serum HBsAg and detectable AF–albumin adducts by radioimmunoassay, among others (30). Informed consent was obtained from each participant before they were randomly assigned to three study groups, and baseline blood and urine samples were collected before the intervention began. Randomization was successful as no significant differences with regard to age, gender and baseline AF–albumin levels were found between groups (30). Participants were instructed to take four capsules daily containing either GTP 500 mg (low-dose, $n = 42$), GTP 1000 mg (high-dose, $n = 41$) or starch 1000 mg as placebo (control, $n = 41$). The doses of 500 and 1000 mg GTP were chosen to be equivalent to two and four cups of tea drink, respectively. Follow-up visits were taken every other day at the participant's house to record possible adverse-effect complaints and to count the remaining capsules for adherence assessment. No severe adverse-effects were recorded according to clinical tests of blood and urine samples at each collection, including blood counts, blood chemistry, alanine aminotransferase (ALT), aspartate aminotransferase (AST), urinary protein, glucose, blood and others (30). An excellent person–time compliance (99.5%) was achieved, and no other consumption of tea or tea products was reported for any participant in this trial (30).

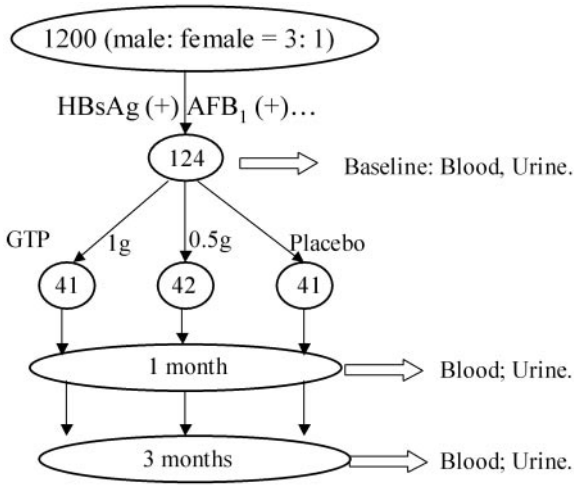

Fig. 1. Overall study design of the phase IIa chemoprevention trial.

In addition to regular epidemiological questionnaires, blood samples (5 ml for serum and 5 ml for plasma) and 24 h urine samples were collected at 1 month and 3 months of the intervention. Serum, plasma and blood cells were immediately separated and stored at −20°C in the village clinics. Twenty-four hour urine samples were collected in the morning, noon and evening in 1 day, and kept in amber bottles containing ascorbic acid (20 mg/ml) and EDTA (0.1 M). Aliquots of urine samples (50 ml) were treated with 500 mg ascorbic acid and 12.5 mg EDTA for analysis of GTP components and 8-OHdG analysis. All samples were shipped frozen to Texas Tech University and the laboratory personnel who performed analysis were blinded to sample sources. This study was approved by Institutional Review Boards of Texas Tech University and Guangxi Cancer Institute for human subject protection. Sample collection, storage and shipment complied with guidelines of both Chinese and US governments.

Analysis of urinary GTP components

The protocol for urinary GTP analysis was modified from methods previously described by Yang *et al*. (29,31). Briefly, thawed urine samples were centrifuged and 1 ml supernatant taken for a 1 h digestion with 500 U of β-glucuronidase and 2 U of sulfatase (Sigma) to release conjugated tea polyphenols. The urine samples were then extracted twice with ethyl acetate. Organic phases were pooled, dried *in vacuo* with a Labconco Centrivap concentrator (Kansas City, MO), reconstituted in 15% acetonitrile, and analyzed with the ESA HPLC-CoulArray system (Chelmsford, MA). The system consists of double Solvent Delivery Modules (Model 582 pump), Autosampler (Model 542) with 4°C cool sample tray and column oven, CoulArray Electrochemical Detector (Model 5600A), and an Operating Computer. The HPLC column was an Agilent Zorbax reverse-phase column, Eclipse XDB-C_{18} (5 μm, 4.6 × 250 mm). The mobile phase included buffer A (30 mM NaH_2PO_4/ACN/THF = 98/1.8/0.2, pH 3.35) and buffer B (15 mM NaH_2PO_4/ACN/THF = 30/63/7, pH 3.45). Flow rate was set at 1 ml/min and the gradient started from 4% buffer B, to 24% B at 24 min, to 95% B at 35 min, kept at 95% until 42 min, dropped to 4% at 50 min, and maintained at 4% until 59 min. Authentic standards were prepared with ascorbic acid and aliquots of the mixture stock were stored at −80°C for 1 month's use. Calibration curves for individual GTP component were generated separately, and EGC, EC, EGCG and ECG were eluted at around 14, 21, 24 and 29 min, respectively. The electrochemical detector was set at −90, −10, 70 and 150 mV potentials, with the main peaks appearing at −10 mV (EGC), 70 mV (EC, EGCG) and 150 mV (ECG). Quality assurance and quality control procedures were taken during analyses, including analysis of authentic standards for every set of five samples and simultaneous analysis of spiked urine sample daily. The limits of detection were 1.0 ng/ml urine for EC and EGC and 1.5 ng/ml urine for EGCG and ECG, respectively. Urinary GTP components were adjusted by creatinine level to eliminate the variation in urine volume.

Analysis of urinary 8-OHdG

Protocol for urinary 8-OHdG analysis was modified from the method described by Renner *et al*. (32). Briefly, 8-OHdG was extracted from 1 ml urine with the Oasis® HLB 3 cc (60 mg) cartridge (Waters) following the manufacturer's instructions. The eluents were dried under ultra-pure N_2 stream and reconstituted in buffer (10 mM ammonium acetate in 2% MeOH, pH 4.3) for analysis with the HPLC-ECD system, which was the same as previously described in the analysis of urinary GTP. The HPLC column for 8-OHdG analysis was Waters YMC basic™ column (S3 μm, 4.6 × 150 mm). The mobile phase consists of buffer A (10 mM ammonium acetate, pH 4.3)

and buffer B (methanol). Flow rate was kept at 0.8 ml/min and a linear gradient (0–40% MeOH in 15 min) was applied for chromatographic separation with the peak of 8-OHdG eluted at ~9.5 min. The CoulArray Detector was set at 270, 300, 330 and 360 mV, with the highest peak appearing at the 330 mV channel. Authentic standard 8-OHdG (Sigma–Aldrich) was used for qualification by retention times and response patterns, and quantification by calibration curves. Similar quality assurance and quality control procedure were applied as described in analysis of urinary GTP, and the limit of detection for 8-OHdG was 1 ng/ml urine. The amount of 8-OHdG was also adjusted by urinary creatinine level for analysis and report.

Analysis of urinary creatinine

Urinary creatinine level was determined colorimetrically with a Diagnostics Creatinine Kit (Sigma–Aldrich) following the manufacturer's instructions. Absorbance at 500 nm was recorded by a DU640 VIS/UV spectrophotometer (Beckman Coulter).

Statistical analysis

Due to the nature of repeated measurements within each participant in this study, the longitudinal data analysis was applied with a multi-level model for change. As only three waves of data were available, a linear change over time was assumed in the following model (33):

$$\text{Level-1 model: } Y_{ij} = \pi_{0i} + \pi_{1i}\text{TIME}_{ij} + \varepsilon_{ij} \quad \varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2)$$

$$\text{Level-2 model: } \pi_{0i} = \gamma_{00} + \gamma_{01}\text{GROUP}_i + \xi_{0i}$$

$$\pi_{1i} = \gamma_{10} + \gamma_{11}\text{GROUP}_i + \xi_{1i} \quad \begin{bmatrix} \xi_{0i} \\ \xi_{1i} \end{bmatrix} \sim N\left(\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma_0^2 & \sigma_{01} \\ \sigma_{10} & \sigma_1^2 \end{bmatrix} \right),$$

where the level-1 model represents the 'within-person change' in biomarker (urinary GTP or 8-OHdG) levels over TIME, and the level-2 model represents the 'between-person differences in change' in biomarker levels and associates the participant's trajectory (intercept and slope) with the predictor, GROUP. The fixed effects, γ_{00}, γ_{01}, γ_{10}, γ_{11}, capture systematic differences according to values of GROUP and are of particular interest here. The model was fitted with MIXED program in SPSS 11.0 (SPSS Chicago, IL) implementing maximum likelihood method for parameters' estimation [(34), http://www.ats.ucla.edu/stat/examples/alda/]. To monitor closely when the efficacy of intervention appears, non-parametric ANOVA (Kruskal–Wallis test) was also applied at all three collections with SPSS to give a cross-sectional perspective of this study. A two-tailed *P*-value <0.05 is reported as significant.

Results

Modulation of urinary GTP biomarkers

Major GTP components in chromatographs were identified and integrated at corresponding maximum-response channels of the CoulArray detector. EGC and EC were readily detected in most samples. The recovery rate with spiked authentic standards at different levels was averaged 75.2 ± 3.9% for urinary EGC and 95.1 ± 1.6% for urinary EC. The coefficient of variance was <13% for EGC and 11% for EC.

Changes in urinary excretion of EGC and EC levels over the baseline, 1 month or 3 month samples after intervention are shown in Figures 2 and 3. The homogeneity of urinary excretion of EGC and EC levels at baseline is demonstrated by cross-sectional ANOVA ($P > 0.80$). Analysis of these urinary GTP data with the multi-level model for change is shown in Table I. The initial level in placebo group (GROUP = 0) was 308.8 ng/mg creatinine for EGC ($\gamma_{00} = 308.8$, $P = 0.000$) and 55.3 ng/mg creatinine for EC ($\gamma_{00} = 55.3$, $P = 0.337$). For GTP-treated groups (GROUP = 500 or 1000 mg), the initial levels of both EGC (γ_{01} un-modeled) and EC ($\gamma_{01} = -0.02$, $P = 0.745$) were not significantly different to placebo group. The slopes for both EGC ($\gamma_{10} = 20.10$, $P = 0.478$) and EC ($\gamma_{10} = -1.08$, $P = 0.976$) in placebo group (see Table I) were not significantly different from 0, which means generally no change in urinary GTP levels in this group were detected over the 3 month period. For GTP-treated groups, however, the slopes were significant. For urinary EGC ($\gamma_{11} = 0.09$, $P = 0.014$), the slope was 0.09 ng/mg creatinine/month higher

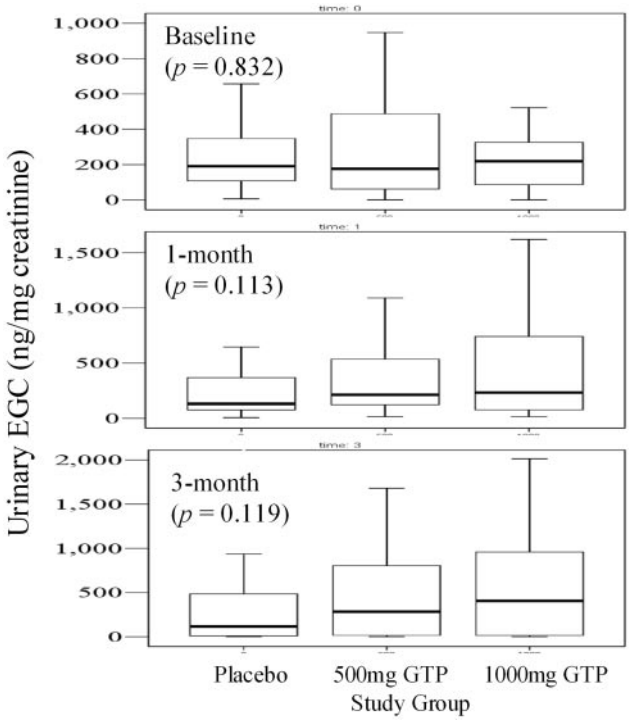

Fig. 2. Urinary excretion of epigallocatechin (EGC). EGC was analyzed for three groups at baseline, 1 month and 3 months. Levels were adjusted by urinary creatinine levels. Upper panel: Baseline EGC levels in three groups show homogeneity ($P = 0.832$). Middle panel: 1 month EGC levels show nearly significant elevation in both GTP-treated groups ($P = 0.113$). Lower panel: 3 month EGC levels remained non-significant but higher in both GTP-treated groups ($P = 0.119$).

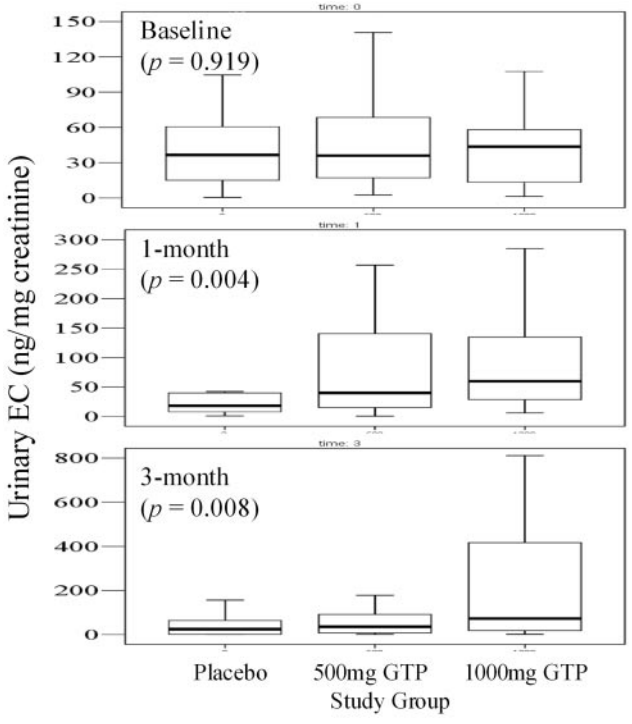

Fig. 3. Urinary excretion of epicatechin (EC). EC was analyzed for three groups at baseline, 1 month and 3 months. Levels were adjusted by urinary creatinine levels. Upper panel: Baseline EC levels in three groups show homogeneity ($P = 0.919$). Middle panel: 1 month EC levels show significant elevation in both GTP-treated groups ($P = 0.004$). Lower panel: 3 month EC levels remained significantly higher in both GTP-treated groups ($P = 0.008$).

Table I. Parameter estimation in multi-level model for biomarker levels

Parameter	EGC	EC	8-OHdG
Intercept, π_{0i}			
Intercept γ_{00}	308.8 ($P = 0.000$)	55.3 ($P = 0.337$)	3.7 ($P = 0.002$)
GROUP γ_{01}	–	−0.02 ($P = 0.745$)	0.002 ($P = 0.277$)
Slope, π_{1i}			
Intercept γ_{10}	20.1 ($P = 0.478$)	−1.08 ($P = 0.976$)	0.524 ($P = 0.384$)
GROUP γ_{11}	0.09 ($P = 0.014$)	0.11 ($P = 0.065$)	−0.002 ($P = 0.036$)

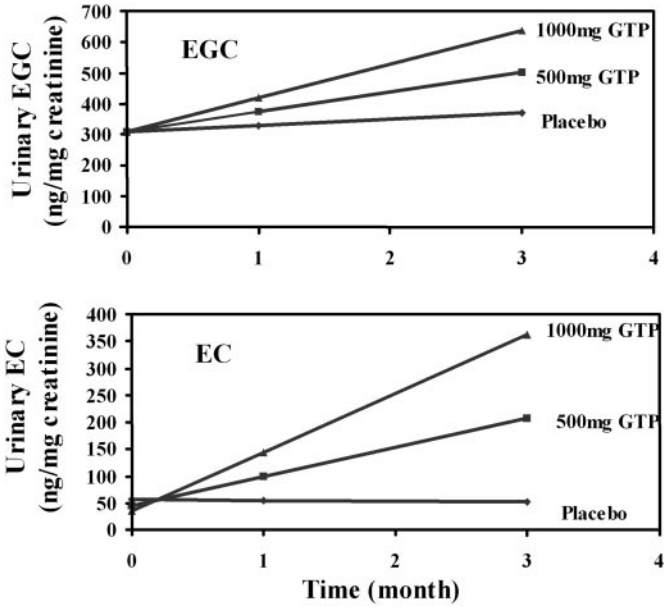

Fig. 4. Prototypical trajectories for urinary GTP levels in three groups. Urinary levels of EGC and EC were fitted to a multi-level model for change with maximum likelihood estimations. Both model fits found no significant change in placebo group over time ($P > 0.470$), but significant and dose-dependent elevation in EGC ($P = 0.014$) and EC ($P = 0.065$) in GTP-treated groups. Upper panel: model estimation of urinary EGC levels in three groups over time with respective prototypical trajectories. Lower panel: model estimation of urinary EC levels in three groups over time with respective trajectories.

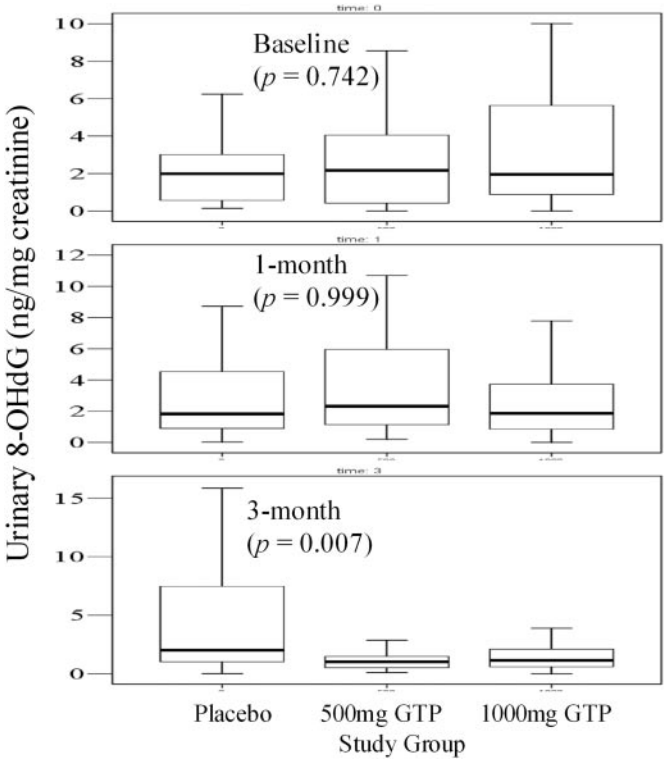

Fig. 5. Urinary excretion of 8-OHdG. 8-OHdG was analyzed for three groups at baseline, 1 month and 3 months. Levels were adjusted by urinary creatinine levels. Upper panel: Baseline 8-OHdG levels in three groups show homogeneity ($P = 0.742$). Middle panel: 1 month 8-OHdG levels remained similar in three groups ($P = 0.999$). Lower panel: 3 month 8-OHdG levels were significantly diminished by GTP intervention ($P = 0.007$).

for every milligram GTP intervention. In two treated groups (GROUP = 500 or 1000 mg), the slope increased 45 ng/mg creatinine/month for the 500 mg group and 90 ng/mg creatinine/month for the 1000 mg group, as compared to the zero slope in the placebo group. For urinary EC ($\gamma_{11} = 0.11$, $P = 0.065$), there is a marginally significant 0.11 ng/mg creatinine/month increase in the slope for every milligram GTP intervention. So the slope of EC increased 55 ng/mg creatinine/month for the 500 mg group and 110 ng/mg creatinine/month for the 1000 mg group, as compared to the zero slope in the placebo group. These results suggest that, while urinary GTP levels remained unchanged in the placebo group over the 3 month period, the GTP-treated groups showed significant, dose-dependent increases in urinary EGC and EC levels over time. The prototypical trajectories in the three groups for both EGC and EC levels were shown in Figure 4. The increased levels of urinary EGC and EC were also found significant at both 1 month and 3 month collection, as shown in Figures 2 and 3.

Modulation of urinary 8-OHdG levels

Several commercially available cartridges, including the LiChrolut EN cartridge, the Waters Sep-Pak cartridge and the Waters Oasis column, were tested for concentration and purification of urinary 8-OHdG. The Waters Sep-Pak (C18 cartridge) can barely retain spiked 8-OHdG in urine. The LiChrolut EN cartridge had a recovery ~50% for spiked 8-OHdG. The notes in the Waters Oasis® HLB (polymer-based column) had a better recovery (>70%) and was tested for its capacity to bind 8-OHdG with a range of 1–6000 ng spiked urine samples and was selected to concentrate and purify 8-OHdG for the analysis of all urine samples. The limit of detection for the Oasis® HLB (60 mg column) was 1 p.p.b. Upon CoulArray detector, 8-OHdG has a maximum response at 330 mV channel, which was chosen to quantify this biomarker in urine samples. Both retention time and response patterns were scrutinized for identifying target peaks, and almost all samples have well-separated and detectable 8-OHdG peaks. The coefficient of variance for the 8-OHdG analyses was <15%.

Changes of urinary excretion of 8-OHdG levels over the baseline, 1 month or 3 month samples after intervention are

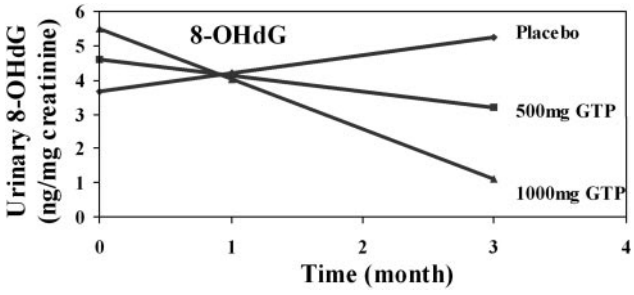

Fig. 6. Prototypical trajectories for urinary 8-OHdG levels in three groups. Urinary levels of 8-OHdG were fitted to a multi-level model for change with maximum likelihood estimations. Fitted model found no significant change 8-OHdG levels in placebo group over time ($P > 0.380$), but significant and dose-dependent level changes ($P = 0.036$) in GTP-treated groups.

shown in Figure 5. The homogeneity of urinary 8-OHdG levels at baseline was again proved by cross-sectional ANOVA ($P > 0.74$). Analysis of urinary 8-OHdG data with the multi-level model for change is also shown in Table I. The initial 8-OHdG level in placebo group (GROUP = 0) was 3.7 ng/mg creatinine ($\gamma_{00} = 3.7$, $P = 0.002$). For GTP-treated groups (GROUP = 500 or 1000), the initial levels ($\gamma_{01} = 0.002$, $P = 0.277$) were not significantly different from the placebo group.

The slope for 8-OHdG ($\gamma_{10} = 0.524$, $P = 0.384$) in the placebo group was not significantly different from 0, suggesting an unchanged 8-OHdG level in this group over the 3 month period. However, for GTP-treated groups ($\gamma_{11} = -0.002$, $P = 0.036$), the slope significantly decreased 0.002 ng/mg creatinine/month for every milligram GTP intervention. For treated groups (GROUP = 500 or 1000), the slope decreased 1.0 ng/mg creatinine/month for the 500 mg group and 2.0 ng/mg creatinine/month for the 1000 mg group, as compared to that in the placebo group. The prototypical trajectories in three groups for 8-OHdG are shown in Figure 6. Though the baseline levels of 8-OHdG seem a little different, they were homogeneous as proved by model fittings ($P = 0.277$) and the slope of placebo group was not statistically different from a zero slope ($P = 0.384$, Table I). These results suggest that, while urinary 8-OHdG levels remained unchanged in the placebo group over the 3 month period, the GTP-treated groups had a significant, dose-dependent decrease in 8-OHdG levels over time. The diminished 8-OHdG levels were also found significant at 3 month collection by cross-sectional ANOVA ($P = 0.007$), as shown in Figure 5.

Discussion

Cancer chemoprevention is defined as the use of drugs, diet or dietary supplements at the earlier stages of carcinogenesis to prevent initiation of cancer, or to retard or delay the progression of cancer (35). In high-risk individuals, initiation is usually assumed, and the goal of chemoprevention is to retard or delay promotion and/or progression. Currently, HCC is the third leading cause of cancer mortality in the world (3–5), and the major etiological risk factors were identified as chronic infection with HBV and dietary exposure to AF (36). While the precise mechanism of HCC formation is poorly understood, one common pathway is the generation of oxidative stress, especially ROS (37,38). To target ROS in HCC high-risk individuals, GTP, a naturally occurring antioxidant, is mechanistically appropriate with well-known safety, efficacy and popularity.

Humans have consumed tea for >5000 years, and currently it is still the most commonly consumed beverage worldwide (11,39). GTP has been shown to inhibit carcinogenesis in the skin, lung, esophagus, stomach, liver, small intestine, pancreas, colon and mammary gland in various animal models, demonstrating its inhibitory activity toward multiple stages of carcinogenesis (14,18). In humans, however, inconsistent results were reported with respect to the role of GTP in cancer risk (13). Lack of objective measures is a common flaw in questionnaire-based investigations where the level of tea ingestion was subjectively classified according to individuals' memories. Urinary biomarkers for tea consumption have practical significances in epidemiological studies and the measurement of various urinary GTP components has been established (25). Validation of these components as biomarkers in high-risk individuals with a controlled GTP intervention, as described in this study, is a high priority in this research field.

In this study, only EGC and EC were readily detected in most urine samples, and were dose-dependent with the dose protocol of intervention, which was consistent with previous reports (25,31,40). Though no tea drinking was reported in the study population, a background level of urinary EGC and EC was detected, which may suggest GTP sources other than tea or tea products in this area, such as vegetables and fruits. Nevertheless, GTP-treated groups, as compared to the placebo group, showed significant and dose-dependent increases in both EGC and EC levels, which supported urinary excretion of EGC and EC as reliable biomarkers for green tea consumptions.

It has been demonstrated that GTP could prevent oxygen free radical-induced hepatocyte lethality and inhibit carcinogen-induced liver oxidative DNA damage (41,42). Humans are ordinarily being attacked by ROS, and DNA constantly damaged (43). The oxidative adduct form of guanine, 8-OH-Gua, is not merely a consequence of oxidative damage, but also a risk factor for further genetic mutations if kept *in situ*. The physicochemical property of 8-OH-Gua affects transcription and replication, and facilitates mispairing with dA and dT (mostly causing G→T substitution) (44,45). 8-OH-Gua also produces base substitution errors at adjacent upstream and downstream template sites (46). Even in the absence of mutations, epigenetic effects have been noticed to affect certain gene expressions: the presence of 8-OH-Gua in promoter elements can affect transcription factor binding, as a single 8-OH-Gua moiety in the promoter region of AP-1 completely prevented transcription factor binding and further gene expression (47).

8-OH-Gua is mainly repaired by base-excisions and the excised product, 8-OHdG, is exclusively excreted into urine (23). Because of its stability in urine (48), its non-invasive sampling, its absence of artifacts as encountered in DNA extraction (24), and more importantly its etiological role in mutations and gene expressions, urinary 8-OH-dG has been proposed to be an appropriate, intermediate biomarker of both oxidative DNA damage and disease outcome (22). In this study, all participants have been exposed to AFB_1 and the community has been chronically infected with HBV for decades, both of which have been reported to increase the formation of ROS in their pathogenic pathways (37,38), thus the oxidative burden in these high-risk individuals is presumably overwhelming the body's defense/repair ability, and an accumulation of massive oxidative DNA damage is expected. This was confirmed by the baseline data with significant

8-OHdG levels in all three groups, ranging from 3.7 to 5.7 ng/mg creatinine. This baseline level was comparable to previously reported 8-OHdG levels in other high-risk populations, such as heavy smokers (24,49). Smokers had a higher level of urinary 8-OHdG, 1.95 μmol/mol creatinine, which corresponds to a level of 4.88 ng/mg creatinine (24). The urinary 8-OHdG level ranged from 5 to 20 ng/mg creatinine for eight male smokers in another report (49). The accumulated oxidative DNA damage seems to be abundant and consistent in study participants, as demonstrated by no significant decreases in 8-OHdG levels after 1 month intervention with GTP. This is different from the previous report that green tea significantly reduced the oxidative burden and decreased urinary 8-OHdG levels in both smokers and non-smokers after 7 days' treatment (50). It seems that combinative oxidative damage caused by HBV and AF is more extensive and more difficult to be modulated. Fortunately, intervention with GTP for 3 months' significantly reduced 8-OHdG levels in these high-risk individuals, which confirmed the efficacy of GTP in chemoprevention of combinative oxidative damage. Previous studies found that GTP modulates body's antioxidant–oxidant balance through changing enzyme profiles, in addition to its free radical-scavenging and metal chelating abilities, e.g. inhibition of oxidative stress-increasing enzymes such as inducible nitric oxide synthase, lipoxygenases, cyclooxygenases and xanthine oxidase, or induction of antioxidant enzymes like glutathione *S*-transferase, glutathione peroxidase, catalase and superoxide dismutase (51). A relatively longer period may be necessary for the induction/inhibition of these enzymes. Significant reduction of 8-OHdG levels after 3 months' intervention, as shown in this study, favors the importance of modulation of enzyme profiles. This will be further examined in the phase III long-term study that is on-going in this high-risk population.

In summary, results of this study suggest that chemoprevention with GTP can effectively reduce 8-OHdG levels, the oxidative DNA damage biomarker. Urinary excretion of EGC and EC was the validated biomarker for green tea consumption.

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