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Validation of Green Tea Polyphenol Biomarkers in a Phase II Human Intervention Trial

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Abstract

Health benefits of green tea polyphenols (GTPs) have been reported in many animal models, but human studies are inconclusive. This is partly due to a lack of biomarkers representing green tea consumption. In this study, GTP components and metabolites were analyzed in plasma and urine samples collected from a phase II intervention trial carried out in 124 healthy adults who received 500- or 1,000-mg GTPs or placebo for 3 months. A significant dose-dependent elevation was found for (-)-epicatechin-3-gallate (ECG) ($p < 0.001$, trend test) and (-)-epigallocatechin-3-gallate (EGCG) ($p < 0.05$, trend test) concentrations in plasma at both 1-month and 3-months after intervention with GTP. No significant increase of (-)-epicatechin (EC) or (-)-epigallocatechin (EGC) was observed in plasma after GTP intervention. A mixed-effects model indicated significant effects of dose (EGCG) and dose by time interaction (ECG), but not for EC and EGC. Analysis of phase 2 metabolic conjugates revealed a predominance of free GTPs in plasma, up to 85% for EGCG, while a majority of GTPs in urine were sulfated and glucuronidated conjugates (up to 100% for EC and 89% for EGC). These results suggest that plasma ECG and EGCG concentrations are reliable biomarkers for green tea consumption at the population level.

Keywords

Green Tea Polyphenols; Biomarker; Intervention; Glucuronidation; Sulfation

1. Introduction

The health benefits of tea and tea extracts have been well documented, especially with respect to chemopreventive effects on cancers (Ahmad and Mukhtar 1999; Yang et al. 2006), cardiovascular diseases (Hollman et al. 1999; Yamada and Watanabe, 2007), and neurodegenerative diseases (Chan et al. 1998). Green tea contains an abundance of polyphenols with anti-oxidative capacities, and the major green tea polyphenols (GTPs) are (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin-3-gallate (EGCG) (Yang et al. 2002). GTPs are strong antioxidants against free radicals (Zhao et al. 2001) and oxidative DNA damage (Luo et al. 2006b), inhibitors of various transcription

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factors such as nuclear factor- κ B (NF- κ B) (Nomura et al. 2000) and activator protein-1 (AP-1) (Chung et al. 1999), and inducers of antioxidant enzymes (Khan et al. 1992) and detoxifying enzymes (Chou et al. 2000). Nevertheless, epidemiological studies in humans have generated inconsistent results regarding the role of tea in cancer risk (Sun et al. 2006a, b). Many studies report reduced risk of carcinogenesis as a result of drinking green tea, while others find no significant association (Lambert and Yang 2003). The situation is further complicated by a few reports of increased cancer risk with tea drinking (Higdon and Frei 2003). Because many epidemiological studies on humans are less controlled than animal experiments, numerous confounding factors could have masked the real effect of GTPs on cancer risk. For example, using questionnaires to estimate dosage of tea consumption is especially prone to bias and imprecision. To overcome this flaw and to reveal the true relationship of GTPs with diseases risks, future epidemiological investigations would benefit greatly from the incorporation of biomarkers of green tea and GTP consumption for a more objective and accurate evaluation, as demonstrated by the Shanghai cohort study (Sun et al. 2002; Yuan et al. 2007).

Biomarkers of GTP consumption have been investigated for more than ten years, with the original forms and simple metabolites of GTP components in human blood and urine samples receiving most attention (Lee et al. 1995). Because they can be obtained less-invasively, biomarkers in blood and urine can readily be adapted into existing and future epidemiological investigations to verify questionnaire-based data with improved precision. Although these GTP biomarkers have been shown to exhibit good dose-response relationships in several individuals (Chow et al. 2001), they have never been validated in human intervention trial. Before applying these biomarkers in epidemiological studies, investigators need to know how precisely they reflect green tea consumption at the population level. Furthermore, GTPs have been reported to go through intensive phase 2 metabolism *in vivo*, including glucuronidation, sulfation, and methylation (Feng 2006; Meng et al. 2002; Vaidyanathan and Walle, 2002). GTP components that are conjugated with glucuronides or sulfate can easily be excised by beta-glucuronidase and sulfatase, respectively. While the two conjugates of GTP have been studied in several subjects with the aid of these enzymes, GTP-conjugation has not been explored at a population-based intervention trial to provide insight into the metabolism profile of tea polyphenols.

In a previous study, we found that urinary excretions of EC and EGC displayed significant and dose-dependent increases in GTP-treated groups, suggesting that urinary EC and EGC can serve as practical biomarkers for green tea consumption in human populations (Luo et al. 2006a,b). Here, we expand our previous study to investigate GTP biomarkers in blood. In addition, the conjugation profiles of GTPs are studied in blood and urine samples to examine the metabolism of GTPs at the population level.

2. Materials and Methods

2.1. Chemicals and instrument

GTP used for the trial was obtained from the US-China joint venture Shili Natural Product Company, Inc. (Guilin, Guangxi, China) and the purity of GTP is higher than 98.5% according to the analysis by Guangxi Standard Bureau. Authentic standards of EC, ECG, EGC, and EGCG, beta-glucuronidase, sulfatase, and tetrahydroxyfuran (THF) were purchased from Sigma Chemical Co. (St. Louis, MO). Methylated EGC and EGCG were kindly provided by Prof. Chung S. Yang from Rutgers University (Piscataway, NJ). Acetonitrile (ACN) was purchased from Burdick & Jackson (Muskegon, MI). Ethyl acetate, methylene chloride, and sodium dihydrophosphate (NaH_2PO_4) were products of Fisher Scientific (Pittsburgh, PA). An ESA HPLC-CoulArray system (Chelmsford, MA) was used for detection of GTP components and metabolites. The system consists of double Solvent Delivery Modules, an Autosampler with 4°C cool sample tray and column oven, a CoulArray Electrochemical Detector (ECD),

and a computer. The reverse-phase column was a Zorbax Eclipse XDB-C18 (5 μ L, 4.6 $\times$ 250 mm) from Agilent Technologies Inc. (Palo Alto, CA).

2.2. Study subjects and study procedure

The overall study design of this phase IIa chemoprevention trial (Figure 1) was described previously (Huang et al. 2004; Luo et al. 2006a,b). Briefly, the study protocol followed NIH standards for chemoprevention trials: randomization, double blindness, and placebo control (USNIH 2000). Twelve hundred adults in southern Guangxi area, China were screened for general-health status and specific biomarkers of hepatitis B virus (HBV) infection and aflatoxin B₁ (AFB₁) exposure. One hundred ninety people (190) with positive HBV surface antigen and serum AFB₁ biomarkers, but with normal liver function consented and were invited to participate in this intervention study. A total of 124 healthy adults aged 20-49 years old were recruited and about one third (41/124) of them were females. They were asked to sign informed consent, randomized into three groups, and treated daily with placebo, 500mg GTP, or 1000mg GTP capsules for 3 months. Each capsule of 250mg GTP contains 126mg EGCG, 53mg ECG, 25mg EC, 19mg EGC, 14mg gallic acid gallate (GCG), and 11mg catechin (C), according to HPLC-ECD and HPLC-UV analysis by Guangxi Standard Bureau and our laboratory in Texas Tech University. The doses of 500 and 1000 mg GTP were chosen to be equivalent to two and four cups of green tea, respectively. The schedule of capsule intake was arranged to be two times a day after a meal. Follow-up visits to the participant's house were made every other day to record possible adverse-effect complaints and to count the remaining capsules for adherence assessment. 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 indicated no severe adverse-effects. 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.

In addition to regular epidemiological questionnaires, blood samples (5 mL for serum and 5 mL for plasma) and 24 h urine samples were collected before the treatment and at 1-month and 3-month after the initiation of GTP intervention. Serum, plasma and blood cells were collected in the morning after an overnight fast and 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 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 the 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.

2.3. Analysis of total plasma GTPs

Protocol for total plasma GTP analysis was modified from an established method (Lee et al. 2000; Meng et al. 2002). Briefly, 200- μ L plasma samples were incubated with beta-glucuronidase (500 units) and sulfatase (40 units) at 37°C for 45 min before repeated treatments with 400 μ L methylene chloride to remove proteins and lipids. The aqueous phases were pooled for double extraction with 700 μ L ethyl acetate, and the organic phase was vacuum-dried and reconstituted for HPLC-ECD analysis. Urine samples were directly extracted and treated with ethyl acetate. The mobile phase of HPLC consists of buffer A (30 mM NaH₂PO₄:ACN:THF at 98%: 1.8%: 0.2%, v/v, pH 3.35) and buffer B (15 mM NaH₂PO₄:ACN:THF at 30%: 63%: 7%, v/v, pH 3.45), and the C18 reverse-phase column was maintained at 35 °C. Flow rate was set at 1 mL/min and a gradient was generated to elute the GTP components and conjugates within 1 h. The 8 channels of the CoulArray detector were sequentially set at -90 mV, -10 mV,

70 mV, and 150 mV, 230 mV, 310 mV, 380 mV, and 450 mV potentials for the detection of GTP components and methylated EGC and EGCG. The main peaks appeared at -10 mV (EGC), 70 mV (EC, EGCG), 150 mV (ECG), and 450 mV (methylated EGC and EGCG). Calibration curves for individual GTP component were generated separately, and EGC, 3'-methylated EGC, EC, EGCG, ECG, and 4', 4''-dimethylated EGCG were eluted at around 14, 16, 21, 24, 29, and 33 min, respectively. Quality assurance and quality control procedures were taken during analyses. These included analysis of authentic standards for every set of five samples and simultaneous analysis of a spiked plasma sample daily. The limits of detection were 0.5 ng/mL plasma for EC, EGC, and methylated-EGC; and 0.5 ng/mL plasma for EGCG, ECG, and dimethylated-EGCG, respectively.

2.4. Analysis of plasma and urinary GTP conjugates

The procedure for analyzing plasma and urinary GTP conjugates was modified from established protocols (Lee et al. 2000; Luo et al. 2006a,b; Meng et al. 2002). To prevent the oxidation of GTP, ascorbic acid and EDTA were added during the extraction process (Luo et al. 2006a,b). The amounts of enzymes, including beta-glucuronidase and sulfatase, and incubation times were optimized in both plasma and urine samples to ensure the release of all conjugates. Plasma and urinary GTP conjugates were then measured under optimal conditions. Briefly, triplicate plasma (200 μ L) samples were incubated without enzyme, with 500-unit beta-glucuronidase, or with 40-unit sulfatase at 37°C simultaneously for 1 h, to generate free GTP, free plus glucuronidated GTPs, free plus sulfated GTPs, and methylated GTPs, respectively. Similarly, triplicate urine (500 μ L) samples were incubated without enzyme, with 200-unit beta-glucuronidase, or with 40-unit sulfatase at 37°C simultaneously for 2 h, to generate free GTP, free plus glucuronidated GTPs, free plus sulfated GTPs, and methylated GTP, respectively. These triplicate-samples were then extracted as described above, vacuum-dried and reconstituted for HPLC-CoulArray analysis. The levels of glucuronidated or sulfated GTP or methylated GTP were calculated by subtraction of free GTP levels from corresponding digestions. The four forms of GTP were pooled as a total for calculation of conjugation percentiles. Similar quality assurance and quality control procedures were taken during analyses as previously described. The limits of detection were 0.5 ng/mL plasma for EC, EGC, and methylated-EGC; and 0.5 ng/mL plasma for EGCG, ECG, and dimethylated-EGCG, respectively.

2.5. Statistical analysis

At each time point, a trend test (nonparametric test for trend across ordered groups) was performed to test the hypothesis that GTPs concentration increased with dose. In addition, the following linear mixed-effects model was used to assess the overall effects of dose and time.

$$Y_{ij} = \beta_1 + \beta_2 \text{DOSE} + \beta_3 \text{TIME}_{ij} + \beta_4 \text{DOSE} * \text{TIME} + b_{i1} + b_{i2} \text{TIME}_{ij} + e_{ij}$$

The intervention period (TIME: 0, 1, and 3 mo), intervention category (DOSE: 0, 500, and 1000 mg-GTPs), and their interaction (Time $\times$ Dose) were examined as fixed effects. Slopes and intercepts of GTP concentration over time within subjects were random effects. This approach is equivalent to the multilevel model approach of Singer and Willett (Singer and Willett 2003), although mixed-effects models allow for more flexible specification of covariance structures. Maximum likelihood was used to estimate parameters with the program MIXED in SPSS 12.0 software (SPSS Inc., Chicago, IL).

Parameter estimates from the above model are robust with respect to deviations from the assumption of normal error distributions; however, we verified results using a nonparametric analysis. GTP concentrations were transformed to ranks, and these ranks were analyzed via the above mixed-effects model (Maas and Hox 2004). Because rank transformations usually

increase heteroscedasticity (Brunner et al. 2002), we allowed the model to estimate separate variances for each group (Singer and Willett 2003). Nonparametric analyses were conducted using R (R Development Core Team 2006) with library nlme (Pinheiro and Bates 2000). To explore GTP metabolism in the population, the conjugated and free forms of GTP within each group were plotted as percentages and analyzed by non-parametric ANOVA. Medians and inter-quartile ranges (IQRs) of the concentrations of different GTP forms also were tabulated to describe the profile of GTP metabolism.

3. Results

3.1. Concentrations of free and conjugated GTP components in plasma

A total of 372 plasma samples from 124 subjects in the three groups were analyzed for the four major GTP components. Trace amounts of total plasma GTPs were found in baseline samples prior to GTP intervention, with EC and ECG concentrations lower than 1 ng/mL, EGC concentrations ranging at 0-7 ng/mL, and EGCG concentrations ranging at 0-4 ng/mL. There were no significant differences between dose groups at baseline of each of these four GTP components ($p>0.28$), with similar dispersion (IQR) among the three groups.

Plasma ECG concentrations showed significant dose-dependent elevation ($p<0.001$, trend test) after GTP intervention (Fig. 2). The averages of ECG after 1-month of intervention were 0.7 ± 0.8 , 5.3 ± 7.3 , and 9.0 ± 9.6 ng/mL and the averages of ECG after 3-month of intervention were 0.3 ± 0.6 , 5.9 ± 9.3 , and 7.6 ± 8.9 ng/mL for the placebo group, low-dose group, and high-dose group, respectively. Significant dose-dependent elevation of plasma EGCG concentrations (Fig. 3) was also found ($p<0.05$, trend test) with the averages at 4.1 ± 4.5 , 9.7 ± 9.4 , and 13.6 ± 12.4 ng/mL after 1-month of intervention and 1.6 ± 1.4 , 5.8 ± 6.9 , and 6.6 ± 10.1 ng/mL after 3-month of intervention for the placebo group, low-dose group, and high-dose group, respectively. On the other hand, plasma EC and EGC concentrations did not show significant elevations after either 1-month or 3-month GTP interventions (Fig. 4 and Fig. 5). As shown in Table 1, significant dose differences (β_2) were found for EGCG and ECG, and a significant dose $\times$ time interaction (β_4) was found for ECG ($p=0.005$). No significant dose or dose $\times$ time interaction was found for EC or EGC concentrations.

Conjugated GTPs were analyzed in 50% of plasma samples randomly selected from each treatment group. The detectable conjugated GTPs include glucuronidated and sulfated EC, ECG, EGC and EGCG. Methylated-EGC or -EGCG was not detectable in these plasma samples under the current analytical protocol. Different profiles of conjugated GTP forms were found and the majority of GTPs in plasma were the free form (Fig. 6).

The free, glucuronidated, and sulfated forms of ECG and EGCG in plasma were expressed as percentages (Fig. 6A). Plasma ECG was mainly the sulfated form (63%) in the low dose group at 1-mo after intervention. However, after increased dosage and/or prolonged intervention time, sulfated ECG in plasma declined to around 40% ($p=0.02$), with a corresponding increase of free plasma ECG from 20% to around 40% ($p=0.005$). Glucuronidated ECG in plasma only occupied 8-16% and showed no significant changes ($p=0.30$) between the low and high doses. On the contrary, EGCG in plasma mainly manifested as the free form (73-85%) (Fig. 6B). Increased dosage or prolonged intervention time did not significantly change the percentages of free EGCG in plasma ($p=0.17$). Glucuronidated and sulfated EGCG occupied comparably low percentages (6-16%) and remained unchanged over different intervention dosages or times ($p>0.17$).

3.2. Concentrations of free and conjugated GTP components in urine

Data on urinary GTPs from this study previously was described in detail (Luo et al. 2006b). Briefly, concentrations of EC and EGC in urine showed significant dose-dependent elevation after GTP intervention; whereas urinary ECG and EGCG concentrations did not show dose-dependent increases after 3-mo intervention.

Conjugated GTPs were also analyzed in 50% of urine samples in which plasma samples were selected for conjugated GTPs analyses. Different from plasma samples, the detectable conjugated GTPs were glucuronidated and sulfated EC and EGC in urine and conjugated GTPs were the apparent form in urine (Fig. 7). Glucuronidated EC was the major form (73-80%) (Fig. 7A), whereas glucuronidated and sulfated EGC was comparable (33-52%) (Fig. 7B). Only smaller portions of the free form of EGC (<16%) and EC (<2.5%) were found in urine samples. Methylated forms of EGC were virtually non-existent. No significant changes were found in these percentiles between dose groups or times with GTPs intervention ($p>0.1$).

4. Discussion

Due to the severe side-effects of synthetic drugs, dietary components and natural products have been considered as a major resource for developing chemopreventive agents against many types of chronic diseases. Among a variety of reported compounds, GTPs have been shown to be relatively safe and effective inhibitors of carcinogen induced mutagenesis and tumorigenesis at several target organ sites, including AFB₁-induced liver tumors in various *in vitro* bioassays and *in vivo* animal models (Ahmad and Mukhtar 1999; Isbrucker et al. 2006; Isbrucker et al. 2006; Isbrucker et al. 2006; Yang et al. 2002). However, human epidemiological studies have so far generated controversial results. Some studies found no association or positive association between tea drinking and cancer risk, while others revealed a reduced risk of cancer in the esophagus, stomach, lung, liver, and prostate with consumption of green tea or GTPs (Bettuzzi et al. 2006; Sun et al. 2006a,b; Yang et al. 2002). A lack of biomarkers representing green tea consumption in questionnaire-based epidemiological studies has hindered the precise evaluation of the possible beneficial health effect of green tea ingestion on human cancer risk. 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 (Yang et al. 1998). However, validating the ability of GTP components in human body fluids to serve as potential biomarkers has not yet been carried out at the population level, and a precise evaluation of the role of GTPs in cancer risk will most likely come from a prospective human intervention study.

In this study, we found that major GTPs, especially EGCG, were detectable in plasma samples after 1- and 3-month of GTP intervention, which is consistent with previous reports with single doses of green tea extracts (Lee et al. 1995; 2002; Yang et al. 1998) or Polyphenon E or EGCG (Chow et al. 2001; Lee et al. 2002) in 4-20 human subjects. We did not find a significant elevation in plasma EC and EGC concentrations after either 1- or 3-month of intervention. EC and EGC were not detected or were present at low/undetectable levels after single-dose administration of EGCG or Polyphenon E in a human phase I pharmacokinetics study (Chow et al. 2001). It seems difficult to make any direct comparison between our data and data published from other studies, because dose protocols used for our and others are totally different. In the single dose study, the plasma levels of EC and EGC were elevated within 1-3 hr after the administration (Lee et al. 1995). In our 3-months repeated-dose study, plasma samples were collected and analyzed after 1- and 3-month treatment.

As previously reported by single dose studies in humans, rats, and mice, EC and EGC are major GTP components conjugated in the liver and rapidly excreted in urine (Chow et al. 2001; Lee et al. 1995; 2002; Li et al. 2000; Sang et al. 2005). We also found that urinary excretions of

EC and EGC displayed significant and dose-dependent increases after 1-or 3-mo of intervention (Luo et al. 2006b), and concentrations of EC and EGC and their metabolites in urine have been used as biomarkers for human cancer studies (Sun et al. 2002; Yuan et al. 2007).

Significant dose-dependent elevation of ECG concentrations in plasma after GTP intervention was found in this study. ECG was not reported in plasma in single dose human studies with green tea extracts (Lee et al. 1995; Yang et al. 1998) or Polyphenon E (Chow et al. 2001), due to the ECG peak was interfered by another compound, which was occasionally seen in baseline samples of this study. Nevertheless, ECG was detectable in plasma of Beagle dogs treated with 200-800-mg/kg body weight per day (Southern Research Institute 2005). In addition to a higher amount of ECG in our GTP preparations as compared to the GTPs used in previous reports, repeated administration may have resulted in elevated levels of ECG, which is distinct from previous studies (Lee et al. 1995; Yang et al. 1998).

Metabolism and biotransformation of GTPs has been well studied in the past 10 years using various *in vitro* and *in vivo* models, including single dosed human samples (Chow et al. 2001; Feng 2006; Lee et al. 1995; 2002; Li et al. 2000; 2001; Lu et al. 2003a,b; Meng et al. 2001; 2002; Sang et al. 2005; Yang et al. 1998). Phase 2 metabolism, including glucuronidation, sulfation, and methylation, has been identified as the major metabolic pathway for GTP components. The role of these conjugations in the long-term biological activity of GTPs largely is unknown. Furthermore, profiles of free vs. conjugated GTP components have not been explored at the population level. In this study, we measured different levels of free and conjugated forms of GTPs in human plasma and urine samples after 3 months of GTP intervention. The majority (73-85%) of plasma EGCG was the free form and free plasma ECG occupied nearly 50% of detectable concentrations in our study subjects. These findings agree with previous reports that the free form accounted for 77% of all EGCG in 8 individuals (Lee et al. 2002), and only a small amount of plasma EGCG was present as conjugates in 20 subjects (Chow et al. 2001). The glucuronidated ECG and EGCG comprised less than 16% in plasma samples. These small but comparable percentages in glucuronidated plasma ECG and EGCG suggest a limited capability for glucuronidation in this population. Sulfated ECG in plasma was found to be the major form (63%) after 1-mo treatment; however, with prolonged treatment up to 3-mo, the relative amount of sulfated plasma ECG decreased, with a corresponding increase in the free form ($p < 0.05$). Thus, free ECG might be important in certain biological activities. On the other hand, only a small amount of plasma EGCG was sulfated, which suggested ECG was preferred as a substrate over EGCG for sulfation in our study subjects.

In contrast to plasma, almost none of the urinary EC (<2.5%), and only a limited amount of urinary EGC (<16%), was found in the free form, generally consistent with a former study where less than 1% of urinary EGC and EC was found in free form (Lee et al. 1995). However, their percentage of glucuronidation was only half that of sulfation in 4 subjects. These results reflect the importance of phase II metabolism in the excretion of EC or EGC, which is also consistent with the lack of a significant elevation of EC or EGC in plasma. It has also been reported that 60-70% of plasma EGC was glucuronidated in 4 individuals (Lee et al. 1995), and most plasma EC and EGC were in the glucuronidation form in 8 subjects (Lee et al. 2002).

A baseline level was observed for all 4 major GTP components in plasma, though the medians could be as low as less than the detection limit (0.5 ng/mL). As no consumption of green tea or other tea products was reported in this population according to the questionnaire analysis, this low baseline level generally reflects a minor absorption or biosynthesis of GTP components from other sources, such as EC in fruits and vegetables. It is known that other polyphenolic compounds are also abundant in vegetables and fruits such as onions, apples, grapes,

blueberries, and grapefruits, and these polyphenols or analogues could have been biotransformed into GTP components *in vivo* in our study subjects. As compared to significantly elevated levels after GTP consumption, this low baseline level does not seem to influence the eventual evaluation of the study outcome. However, it does suggest that the normalization of background GTP component levels is necessary to avoid potential confounding effect in future epidemiological studies using these components as biomarkers. Furthermore, the levels of total GTPs, especially ECG and EGCG, in plasma decreased at samples of 3-month compared to those collected at 1-month post intervention, which may reflect individual variations in bioavailability or excretion rate of these components or seasonal fluctuations of their pharmacokinetics and metabolism.

In summary, our results suggest that plasma ECG and EGCG and urinary EC and EGC may be used as reliable biomarkers to reflect the consumption of green tea or GTP supplements at the population level. These results would be helpful in future epidemiological studies to further investigate the role of GTPs in chronic diseases in humans.

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Abbreviations

GTPs	green tea polyphenols
EC	(-)-epicatechin
ECG	(-)-epicatechin-3-gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin-3-gallate
HCC	hepatocellular carcinoma
IQRs	

inter-quartile ranges

Fig. 1.
Study Design of Phase II Chemoprevention Trial with GTP.

Fig. 2.

Total plasma ECG level in the three groups at each collection. A total of 372 plasma samples were analyzed. The averaged concentrations of plasma ECG at baseline were 0.5 ± 0.5 ng/mL, 2.1 ± 9.4 ng/mL, and 0.5 ± 0.9 ng/mL for the placebo, 500mg GTP, and 1000mg GTP groups, respectively, and showed dose-dependent and significant elevation at 1-month and 3-month collections as described in detail in Results. A Trend test was performed for the statistical analysis. The box plot represents outliers, 5%, 25%, median, 75%, and 95%, respectively.

Fig. 3.

Total plasma EGCG level in the three groups at each collection. A total of 372 plasma samples were analyzed. The average concentrations of plasma EGCG at baseline were 3.4 ± 2.6 , 4.9 ± 8.1 , and 3.6 ± 2.7 ng/mL for the placebo, 500mg GTP, and 1000mg GTP groups, respectively, and showed dose-dependent and significant elevation at 1-month and 3-month collections as described in detail in Results. A Trend test was performed for the statistical analysis. The box plot represents outliers, 5%, 25%, median, 75%, and 95%, respectively.

Fig. 4.

Total plasma EC level in the three groups at each collection. A total of 372 plasma samples were analyzed. The averaged concentrations of EC at baseline were 0.4 ± 0.3 , 0.3 ± 0.2 , and 0.4 ± 0.2 ng/mL; after 1-month of intervention were 0.6 ± 0.4 , 0.5 ± 0.3 , and 0.7 ± 0.5 ng/mL; and after 3-month of intervention were 0.8 ± 0.4 , 0.9 ± 0.5 , and 0.8 ± 0.3 ng/mL for the placebo, 500mg GTP, and 1000mg GTP groups, respectively. A Trend test was performed for the statistical analysis. The box plot represents outliers, 5%, 25%, median, 75%, and 95%, respectively.

Fig. 5.

Total plasma EGC level in the three groups at each collection. A total of 372 plasma samples were analyzed. The averages of EGC at baseline were 6.5 ± 2.3 , 6.9 ± 4.5 , and 6.3 ± 2.6 ng/mL; after 1-month of intervention were 5.2 ± 7.3 , 3.9 ± 5.1 , and 6.1 ± 6.9 ng/mL; and after 3-month of intervention were 4.6 ± 3.5 , 4.8 ± 3.5 , and 5.1 ± 4.2 ng/mL for the placebo, 500mg GTP, and 1000mg GTP groups, respectively. A Trend test was performed for the statistical analysis. The box plot represents outliers, 5%, 25%, median, 75%, and 95%, respectively.

Fig. 6. Average percentages of different forms of plasma ECG (A) and EGCG (B) in the selected groups and collections. A total of 186 plasma samples were analyzed.

Fig. 7. Average percentages of different forms of urinary EC (A) and EGC (B) in the selected groups and collections. A total of 186 urine samples were analyzed.

Table 1
Parameter estimates in the mixed-effects model for plasma GTP concentrations*

Parameter	EC	ECG	EGC	EGCG
Intercept	β_1	1.028 (p=0.193)	6.066 (p=0.000)	4.346 (p=0.000)
Dose	β_2	0.002 (p=0.057)	0.001 (p=0.836)	0.003 (p=0.029)
Time	β_3	-0.051 (p=0.915)	-0.603 (p=0.055)	-0.663 (p=0.208)
Dose×Time	β_4	0.002 (p=0.005)	0.001 (p=0.736)	0.001 (p=0.147)

* Plasma concentrations of GTP components were fit with a mixed-effects model assuming a linear trajectory. Model parameters were estimated using maximum likelihood.