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Green tea polyphenol suppresses tumor invasion and angiogenesis in N-butyl-(-4-hydroxybutyl) nitrosamine-induced bladder cancer

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ABSTRACT

Background: Green tea polyphenol (GTP) suppresses malignancy in bladder cancer cell lines. However, the detail of its anti-carcinogenic effect *in vivo* is not fully understood. This study investigated the effect of GTP on bladder tumor size and angiogenesis in mice given N-butyl-(-4-hydroxybutyl) nitrosamine (BBN), with and without GTP. *Methods*: Eight-week-old female C3H/He mice were treated with and without 0.05% BBN solution for 14 or 24 weeks. In addition, they were also treated with and without 0.5% GTP solution for the same periods. Histopathological diagnosis was established using hematoxylin and eosin staining, and microvessel density (MVD) was estimated by counting CD34- and von Willebrand factor-positive vessels in the tumor area. *Results*: At 14 weeks, cancer cells were detected in BBN and BBN + GTP mice [5/14 (35.7%) and 3/14 (21.4%), respectively, $p = 0.678$]. At 24 weeks, the incidence of cancer cells was also similar between the groups (BBN + GTP: 61.9% vs. BBN: 82.6%; $p = 0.179$). However, the frequency of invasive tumors in BBN + GTP mice was significantly lower (23.8%; $p = 0.030$) than in those given BBN alone (65.2%). Tumor volume and MVD of intratumoral and stromal region in the BBN + GTP group were also significantly lower than in BBN mice. *Conclusion*: The results showed that GTP had no anti-carcinogenic effect, but inhibited tumor growth and invasion in mice with established bladder cancer, at least in part through the regulation of angiogenesis. Our data suggest that GTP seems to suppress tumor development in bladder cancer.

1. Introduction

Bladder cancer is one of the most common malignancies worldwide especially in industrialized countries. The mortality rate of bladder cancer is anticipated to increase due to exposure of more people in the developing countries to various chemical agents by industrial development and changes in eating habits. At diagnosis, approximately three-quarters of bladder cancers include superficial tumors confined either to the epithelium or to the lamina propria [1]. Such cases are usually treated by transurethral resection and/or adjuvant intravesical treatments with either chemotherapy or Bacillus Calmette-Guerin (BCG vaccine), resulting in a reasonable prognosis. However, even at such an early stage, bladder cancer cells often invade the surrounding muscle layer after initial treatment [2]. Invasion of adjacent tissue is a crucial step in the systemic dissemination of any cancer and subsequent appearance of distant metastasis. Once bladder cancer progresses to this stage, the prognosis becomes poor despite further treatments such as radical cystectomy, chemotherapy, and radiation therapy. In addition, these treatments may cause side effects and significantly decrease quality of life (QOL). Therefore, prevention of tumor growth and invasion helps maintain QOL and enhances prognosis of patients with superficial bladder cancer.

Many studies centered on the development of anti-tumor agents and investigated the mechanisms underlying tumor progression both *in vitro* and *in vivo*. The polyphenolic compounds in green tea (green tea polyphenol, GTP) reportedly have anti-carcinogenic and chemopreventive effects on various malignancies [3–5], including bladder cancer, where similar results were obtained in cancer cell lines and animal models [6–9]. However, opinion is divided regarding the anti-carcinogenic effect of drinking green tea. In addition, although several molecules have been associated with the anti-tumor effects of GTP, there are insufficient data for a detailed mechanism to be devised [7,8,10]. For instance, the relationship between GTP intake and angiogenesis is not completely understood, and angiogenesis is an important factor for bladder cancer progression.

The present study sought to clarify the preventive effects of GTP on carcinogenesis, tumor growth, and muscle invasion in bladder cancer. It also examined the relationship between GTP intake and

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intratumoral angiogenesis. We used an N-butyl-N-(-4-hydroxybutyl) nitrosamine (BBN)-induced bladder cancer mouse model. This animal model is a recognized method of investigating the invasive potential of bladder cancer because invasive tumors can develop within days of the induction [11]. This model has been used previously in various animal studies of bladder cancer [12–14].

This is the first examination of the relationship between GTP intake and angiogenesis in BBN-induced bladder cancer. The results indicated that GTP inhibited tumor growth and invasion, but not carcinogenesis, through the suppression of angiogenesis. These results could broaden the treatment strategies available for patients with bladder cancer.

2. Materials and methods

2.1. Mouse BBN-induced bladder cancer model

N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) and GTP were obtained from Tokyo Kasei Industries (Tokyo, Japan) and LKT Laboratories, Inc. (St. Paul, MN), respectively. Female C3H/He mice were obtained from Japan Charles River, Yokohama, Japan at 6 weeks of age and housed in polycarbonate cages (2 or 3 mice per cage) in a controlled-environment room at 22 °C on a 12-h light/12-h dark cycle. The study was limited on female mice for the following two reasons. (1) Male mice are more likely than females to develop bladder cancer induced by BBN [15]. In addressing this phenomenon, a recent study demonstrated that androgen and its receptor are associated with tumor growth in BBN-induced bladder cancer in mice [16]. Because GTP reduces the activity of androgen receptor signaling in mice [4], we were concerned that the anti-tumor effects of GTP in a BBN-induced bladder cancer model using male and female mice may reflect regulation of androgen and its receptor, and not an anti-angiogenic function. (2) The age-standardized mortality rate (ASR) of bladder cancer in men reached a plateau many years ago and then decreased in the 1990s [17]. In contrast, the ASR of bladder cancer in females increased slightly after 1993. Taken together, these factors justified establishing a female-mouse model of BBN-induced bladder cancer in this study.

A 0.05% concentration of BBN in tap water in dark bottles was supplied *ad libitum* to 8-week-old mice for 14 and 24 weeks. With regard to GTP, 0.5% concentration solution was provided for similar periods. Negative control mice received tap water or GTP solution alone throughout the experiment. The drinking water was prepared fresh twice a week. The volume of water intake was 404–432 and 702–738 ml during the 14- and 24-week period, respectively. There was no significant difference in the amount of consumed water between the two groups (data not shown). The calculated range of GTP intake during the 14 and 24 weeks was 2.02–2.16 and 3.51–3.69 g, respectively. The mice were sacrificed at 15 and 25 weeks following the first BBN treatment. In this study, mice were divided into 8 groups according to the content of drinking water and periods after BBN treatment (Table 1). Thus, mice that were sacrificed at 14 weeks were divided into four groups: tap water group 1, GTP solution group 2, BBN group 3, and BBN + GFP group 4. Mice sacrificed at 24 weeks were divided into four similar groups (groups 5–8). Based on preliminary results, we used 15 and 23 mice in each of groups 1–6 and groups 7 and 8, respectively. In groups 3 and 4, one mouse died at 2–3 weeks. In addition, 2 mice from group 8 were also dead at 3 weeks. All animals in this study were handled and treated according to the Guidelines for Animal Experiments of Nagasaki University, and the Regulations of Animal Care and Use committee approved the study protocol.

2.2. Tissues and immunohistochemistry

After euthanasia, the mouse urinary bladders were filled with phosphate-buffered 10% formalin and then immersed in the fixative with the bladder neck closed for 24 h. The fixed specimens were processed for embedding in paraffin. The tissue blocks were then cut into sections every 100 μm and subjected to routine histological examination after staining with hematoxylin and eosin. Tumor volumes were calculated as $(L \times S^2)/2$, where L (mm) is the largest diameter and S (mm) is the smaller diameter. When pleural tumors were found, their volumes were averaged for analyses.

We also used the paraffin sections for immunohistochemical staining of CD34 and von Willebrand factor (vWF). Tissue sections (5-μm thick) were deparaffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval was performed at 95 °C for 40 min in 0.01 M sodium citrate buffer (pH 6.0). All sections were then immersed in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Sections were incubated with a protein-blocking solution containing pre-immune serum and then with the primary antibody (anti-CD34; HM1015, Hycult Biotechnology, The Netherlands, and anti-vWF; AB7356; Chemicon International Inc., Temecula, CA) at 4 °C overnight. The sections were washed in 0.05% Tween 20 in phosphate-buffered saline (PBS), and then incubated with peroxidase-conjugated anti-rabbit IgG (Dako Corp, Carpentaria, CA) for 60 min. The peroxidase reaction was visualized with a liquid 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Zymed Laboratories). Sections were counterstained in hematoxylin, dehydrated through graded alcohol solutions, and cleared in xylene before mounting using Polymount (Polysciences, Warrington, PA). A consecutive section from each sample was processed without the primary antibody as a negative control, and kidney tissue was used as the positive control. MVD was measured in tumor sections with the largest diameters.

2.3. Tumor microvessel density

The microvessel density (MVD) represented the average number of CD34- and vWF-stained vessels in the intratumoral

Table 1
Incidences of cancer and cancer invasion in the eight mice study groups at 14 and 24 weeks.

Group (duration)	BBN intake	GTP intake	*n*	Incidence of cancer (%)	Incidence of invasion (%)
14 weeks					
1	–	–	15	0 (0)	0 (0)
2	–	+	15	0 (0)	0 (0)
3	+	–	14	5 (35.7)	1 (7.1)
4	+	+	14	3 (21.4)	0 (0)
24 weeks					
5	–	–	15	0 (0)	0 (0)
6	–	+	15	0 (0)	0 (0)
7	+	–	23	19 (82.6)	15 (65.2)
8	+	+	21	13 (61.9)	5 (23.8)

BBN: N-butyl-(-4-hydroxybutyl) nitrosamine, GTP: green tea polyphenol.

and tumor-stromal areas, as described previously [18]. To estimate MVD, sections were scanned at low magnifications (×40 and ×100) to identify 2–3 'hot-spot' areas with the highest prevalence of positively stained vessels. Each hot spot was then further examined at ×200 magnification using Nikon E-400 microscope and representative images were taken using a digital camera (Nikon DU100 camera, Tokyo) and subjected to image analysis (Win ROOF, Version 5.0, MITANI Corp, Fukui, Japan). The MVD was defined as the number of vessels per high power field (HPF, ×200). We also used a similar imaging analysis system to measure tumor diameter.

2.4. Statistical analysis

All data were expressed as median and interquartile range (IQR) due to the skewed distribution patterns. The Mann–Whitney *U* test was used to compare continuous variables. The chi-square test was used for categorical comparison of the data. Survival analysis was evaluated by Kaplan–Meier analysis and the log-rank test. Variables that achieved statistical significance ($p < 0.050$) by univariate analysis were subsequently analyzed by multivariate analysis using a COX proportional hazards analysis (described as odds ratios [ORs] with 95% confidence intervals [95%CIs], together with the *p* values). The crude and adjusted effects were estimated by logistic regression analysis. All statistical analyses were two-sided and significance was defined as $p < 0.050$. All statistical analyses were performed on a personal computer with the statistical package StatView for Windows (version 5.0, Abacus Concept, Berkeley, CA).

3. Results

3.1. Inhibitory effects of GTP on carcinogenesis and invasion

The mean body weight was similar among the BBN intake and control groups during the experimental period. Table 1 details the percentage of cancer in the BBN-induced murine bladder cancer model. The tap water (groups 1 and 5) and GTP (groups 2 and 8) mice showed normal histology, regardless of the period of intake. In mice sacrificed after 14 weeks of BBN treatment, cancer cells were detected in 35.7% and 21.4% of group 3 (BBN) and group 4 (BBN + GTP), respectively. There was no significant difference ($p = 0.678$) in frequency between these two groups. In contrast, after treatment for 24 weeks, bladder cancer cells were detected in 82.6% and 61.9% of mice in groups 7 (BBN) and 8 (BBN + GTP), respectively. However, this still did not reach significance ($p = 0.179$). We found invasive bladder cancer in 65.2% of group 7 mice, while only one mouse in group 3 showed invasive tumor (equivalent groups at 24 and 14 weeks, respectively). In addition, this frequency in group 7 mice was significantly higher than that in group 8 (23.8%, Table 1). Five and 1 tumors were judged as to have invaded the surrounding tissue in groups 7 and 8, respectively.

3.2. Tumor volume and MVD

Fig. 1 shows representative examples of BBN-induced bladder cancer in group 3 (a) and group 7 (b), while Fig. 2a summarizes tumor volumes in groups 7 and 8. At 24 weeks, the median (IQR) tumor volume in group 7 (0.81, 0.41–1.31 mm^3) was significantly

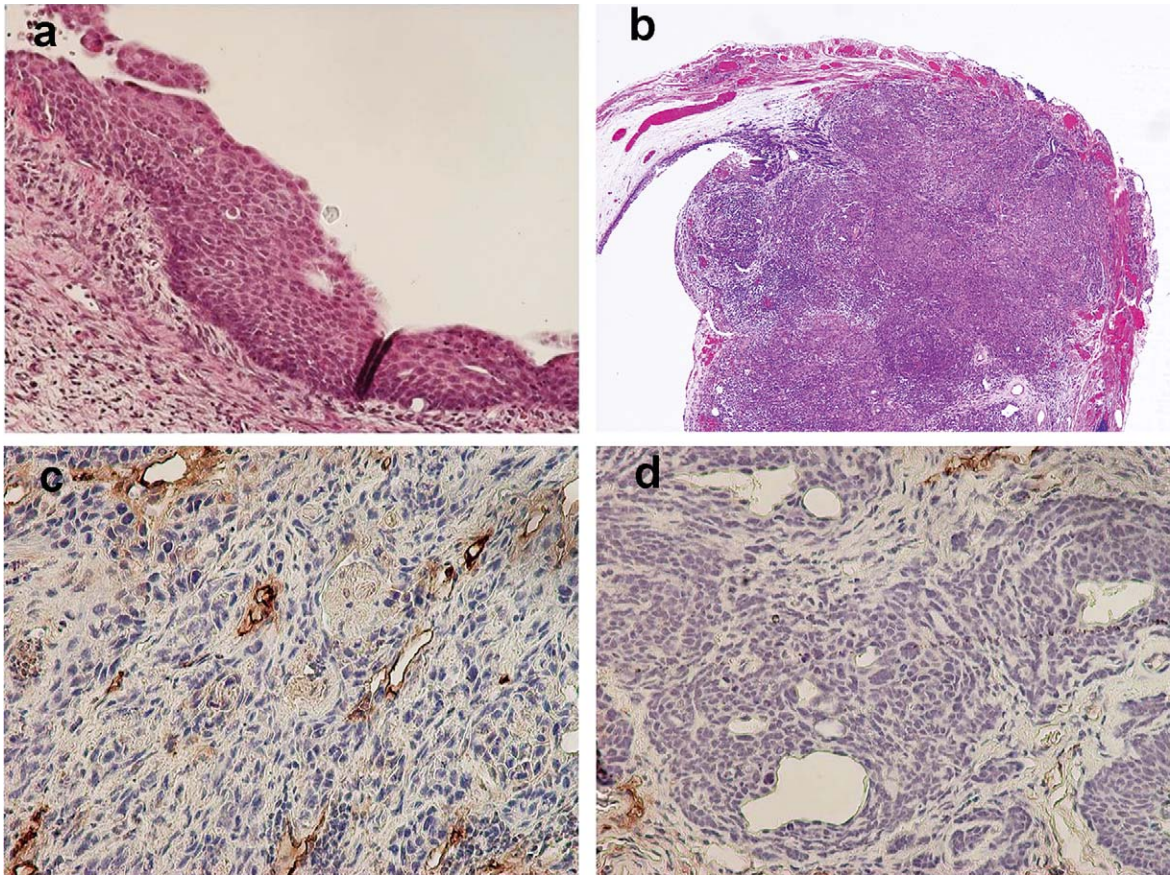

Fig. 1. Hematoxylin and eosin staining of BBN-induced bladder cancer in representative mice of group 3 (BBN intake for 14 weeks) and group 7 (BBN intake for 24 weeks). Group 3 showed a thickened mucosal layer and presence of cancer cells, although no cancer cell invasion was detected (a, magnification ×100). Large tumors and cancer cell invasion into muscle layers were observed in group 7 (b, magnification ×40). Representative examples of vWF-positive vessels in an invasive tumor from group 7 (BBN for 24 weeks) and group 8 (BBN + GTP for 24 weeks) mice (c, magnification ×200 and d, magnification ×200, respectively). Although these two tumors were of similar volume, the number of vWF-positive vessels in group 8 was clearly lower than in group 7.

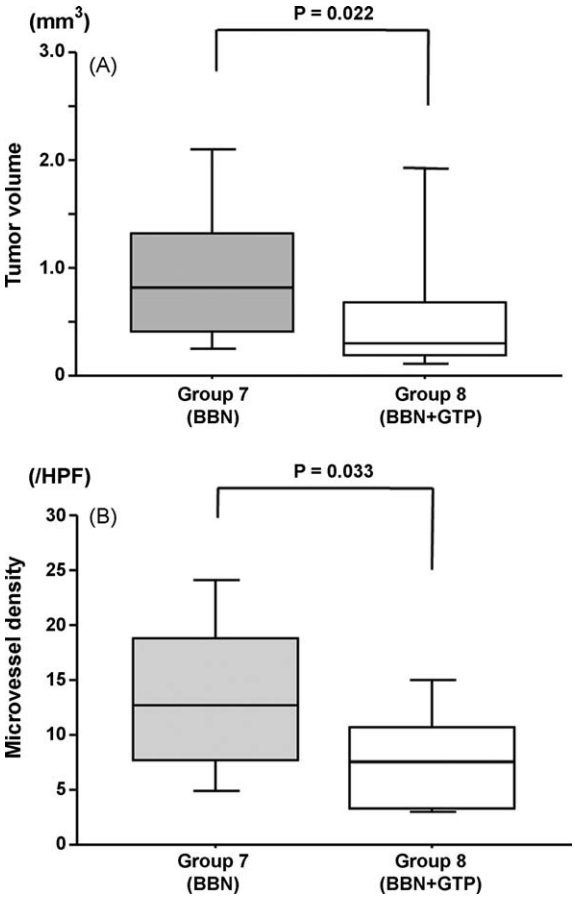

Fig. 2. Box-and-whisker plots of tumor volume (a) and intratumoral microvessel density (iMVD, b) in group 7 (BBN intake only) and group 8 (BBN + GTP intake) mice. In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. Tumor volume and iMVD were significantly lower in group 8 mice than in those of group 7.

larger than that in group 8 (0.29, 0.18–0.67 mm^3). Group 7 mice also showed vWF-positive vessels in all specimens (Fig. 1c), while such vessels were rare in intratumoral areas in group 8 mice, regardless of the invasive state or size of the tumor (Fig. 1d). No mice in the tap water or GTP control groups (groups 1, 2, 5, and 6) showed vWF-positive vessels in urothelial epithelia, although urothelial CD34-positive vessels were detected; only two specimens in group 3 and one in group 4 showed CD-34-positive vessels at 14 weeks. We therefore only calculated MVD for groups 7 and 8. The MVD value of group 8 (7.5, 3.2–10.7/HPF) was significantly lower than that of group 7 (12.7, 7.6–18.8/HPF, Fig. 2b). Furthermore, MVD correlated positively and significantly with tumor size in the 24-week treatment groups ($r = 0.493$, $p < 0.001$).

4. Discussion

The results presented here demonstrated that GTP intake did not inhibit carcinogenesis in BBN-induced bladder cancer of female mice. A previous study reported that green tea intake prevented bladder cancer but the study was conducted in a rat model of BBN-induced bladder cancer, and not in mice [6]; this is important since the malignant potential is known to differ in these animals [19]. In addition, the rat model might not be suitable for investigating invasive mechanisms and the anti-invasion effects of GTP because almost all tumors in the rat model were of the superficial type [20]. To our knowledge, there is no report of GTP inhibiting carcinogenesis in a mouse BBN-induced bladder cancer model. Several epidemiological studies in Japan and Taiwan have addressed the relationship between green tea intake and bladder cancer risk because green tea is one of the most frequently consumed beverages in Asia [21–23]. However, no link was established between increased green tea intake and prevention of carcinogenesis in bladder cancer. The concentration of GTP used in these animal models is equivalent to that in the average intake of green tea in humans. However, we speculate that the anti-carcinogenic effect of GTP is not always experimentally significant, at least *in vivo*.

The present study found that the intake of GTP for 24 weeks inhibited tumor growth and cancer cell invasion in mice with induced bladder cancer. Several molecular components and signaling pathways have been implicated in the anti-tumor mechanism underlying the observed effect of GTP in bladder cancer, including N-cadherin [8], Akt signaling [7,8], the Bcl-2 family of proteins [7], and cell-cycle components [10]. However, there is no information on the anti-angiogenic effect of GTP in animal bladder cancer models, as evaluated by MVD. The present results demonstrated that iMVD in mice administered BBN + GTP was significantly lower than in those given only BBN group at 24 weeks, and that iMVD correlated to tumor volume. In other studies, GTP inhibited angiogenesis via modulation of endothelial cell proliferation, migration, and tube formation *in vitro* [24,25]. Given the importance of angiogenesis to the growth of solid tumors including bladder cancer, we speculate that GTP could inhibit tumor growth via regulation of angiogenesis in bladder cancer tissues.

A salient feature of this study is the sole use of female mice. The incidence of BBN-induced bladder carcinoma could vary widely in mice due to several factors including age, duration of drinking, breed of mouse, and gender. Miyamoto et al. [16] reported that the incidence of BBN-induced bladder carcinoma in female mice at 20 and 30 weeks of age was 0% (duration of BBN intake = 12 weeks). On the other hand, Hikosaka et al. [26] reported that 29% of 26–28-week-old mice showed BBN-induced bladder carcinoma (duration of BBN intake = 10 weeks), and that the percentages were lower in female mice than in male mice despite equivalent experimental conditions. Our original study design was to use male mice. However, the effect of GTP on regulation of androgen and androgen receptor function might have complicated any anti-tumor effects. Thus, female mice were chosen for analysis of the anti-angiogenic effects of GTP as accurately as possible.

In the present study, BBN solution was given for the longest duration in comparison to previous reports. We also used C3H/He mice, which are highly susceptible to chemically induced carcinoma [27]. Although it remains unclear how such factors affect the tumoral activities, our experimental animal model was deemed the most suitable for analyzing the malignant potential and behavior of BBN, and the anti-tumoral effect of GTP.

Based on various experiments using cancer cell lines, there is no doubt that GTP has anti-tumoral effects in bladder cancer. However, there is no proof so far that increased green tea intake reduces the risk and progression of bladder cancer in human [28]. Also, there is a limit to the possible daily intake of GTP. Thus, further pharmacological studies and clinical trials are needed to confirm the anti-tumoral effects of GTP. The present results provide valuable information for the design and patient selection in clinical trials of GTP as an anti-tumor agent in bladder cancer.

In conclusion, the present study suggested that GTP intake could inhibit the growth and invasive of tumors in a female-mouse bladder cancer model. In addition, such anti-tumoral effects seem be at least in part regulated through the inhibition of angiogenesis. Based on these results, we speculate that GTP intake may help prevent muscle invasion and cancer cell progression in patients with superficial bladder cancer.

Conflict of interest

None of the authors has any relationship with other individuals, organizations, and companies that could inappropriately influence the work reported in this study. This original study was not supported financially by any funding source.

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