Jpn. J. Cancer Res. 85, 221–225, March 1994

Inhibitory Effects of (−)-Epigallocatechin Gallate on Spontaneous Hepatoma in C3H/HeNCrj Mice and Human Hepatoma-derived PLC/PRF/5 Cells

Hiroshi Nishida,[1] Masahide Omori,[2] Yasushi Fukutomi,[1] Mitsuo Ninomiya,[1] Shinji Nishiwaki,[1] Masami Suganuma,[3] Hisataka Moriwaki[1] and Yasutoshi Muto[1,4]

[1]*First Department of Internal Medicine, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500,* [2]*Tokai Women's College, Research Institute of Bio-Science, Nakakirino, Kakamigahara 504 and* [3]*Saitama Cancer Center Research Institute, Ina, Kitaadachi-gun, Saitama 362*

The inhibitory effect of (−)-epigallocatechin gallate (EGCG), a main constituent of Japanese green tea, on spontaneous hepatoma in C3H/HeNCrj mice was investigated. A total of 72 mice were divided into three groups; the control group without EGCG, and two experimental groups receiving 0.05% (w/w) or 0.1% EGCG in drinking water. EGCG reduced the incidence of hepatoma-bearing mice from 83.3% (control) to 56.0% (0.05% EGCG) and 52.2% (0.1% EGCG), and also reduced the average number of hepatomas per mouse from 1.83 (control) to 0.72 (0.05% EGCG) and 0.91 (0.1% EGCG) at week 65. Ridit analysis of the distribution of the number of hepatomas in each group revealed that EGCG significantly increased the rate of mice without hepatoma in the two EGCG groups as compared to the control. EGCG did not affect body weight gain, food consumption or any serum biochemical parameter. EGCG inhibited the growth and secretion of α-fetoprotein by human hepatoma-derived PLC/PRF/5 cells without decreasing their viability. These results indicate that EGCG may be a practical, nontoxic preventive agent against human hepatoma.

Key words: (−)-Epigallocatechin gallate — Spontaneous hepatoma — C3H/HeNCrj mouse — Green tea — PLC/PRF/5 cell

It has recently been proposed that the development of human cancers could be inhibited by taking certain substances contained in daily foods or drinks. Green tea is a candidate, in view of epidemiological evidence of low standardized mortality rates for all forms of cancer in the population of the Shizuoka area of Japan, where people drink large amounts of green tea.[1)] Decreased risks of stomach cancer and urinary bladder cancer were also reported to be associated with high consumption of green tea.[2,3)] Green tea extract and some components of green tea are known to inhibit carcinogenesis in the skin, lung, esophagus, forestomach and colon in several rodent models.[4–9)] EGCG[5] (Fig. 1) is a polyphenolic main constituent of Japanese green tea and has the strongest antioxidant activity among the catechins in the leaf.[10)] EGCG inhibited the tumor-promoting activity of both teleocidin, one of the 12-*O*-tetradecanoylphorbol-13-acetate-type tumor promoters, and okadaic acid in two-stage carcinogenesis experiments on mouse skin,[11,12)] and also inhibited mouse duodenal carcinogenesis induced by N-ethyl-N′-nitro-N-nitrosoguanidine.[13)] Since administration of green tea extract containing EGCG significantly inhibited the labeling index of preneoplastic foci in mouse liver treated with phenobarbital,[14)] we expected that carcinogenesis in the liver would also be prevented by EGCG.

In this report, we demonstrate that EGCG inhibits spontaneous liver tumors in C3H/HeNCrj mice without showing any toxicity. In addition, EGCG inhibited the growth and secretion of AFP by human hepatoma-derived PLC/PRF/5 cells. The possible role of EGCG as a cancer-preventive agent, as exemplified by its ability to prevent human hepatoma, is discussed in the present paper.

MATERIALS AND METHODS

Animals and chemicals Eight-week-old male C3H/HeNCrj mice were purchased from Charles River Japan Inc. (Atsugi). EGCG was isolated from Japanese green tea as reported previously.[11)] The isolate was composed of 85% EGCG, 10% (−)-epigallocatechin and 5% (−)-epicatechin gallate.

Animal experiment in C3H/HeNCrj mice All mice were acclimatized to our facilities for the initial 6 weeks. The mice were then randomly divided into three groups: group A (control group, 20 mice), group B (0.05% EGCG, 26 mice) and group C (0.1% EGCG, 26 mice). Groups B and C were given tap water containing 0.05% and 0.1% EGCG, respectively, *ad libitum* from week 7 of the experiment (14 weeks of age), while group A was given tap water without EGCG. The mice were housed

[4] To whom correspondence should be addressed.

[5] The abbreviations used are: AFP, α-fetoprotein; EGCG, (−)-epigallocatechin gallate.

in plastic cages (25.5×41.5×15.5 cm) with wood chip bedding in an air-conditioned room at around 25°C and fed a commercial pelleted diet (MF: Oriental Yeast Co., Tokyo) *ad libitum* for the entire experimental period. Their body weight and food consumption were measured every week. At week 65 (72 weeks of age), all surviving mice were killed under ether anesthesia and blood was drawn from the carotid artery for analyses of serum albumin, total bilirubin, glutamic pyruvic transaminase, γ-glutamyl transpeptidase, and total cholesterol by an autoanalyzer.[15] Following laparotomy, liver tumors were counted and the diameter of each was measured. Liver tissues were fixed in formalin, embedded in paraffin, and subjected to hematoxylin and eosin staining for histological analysis. Hepatomas were classified according to Frith and Ward as one of two types: hepatocellular adenoma and hepatocellular carcinoma.[16] Statistical analyses were performed using Fisher's exact test to compare tumor incidence and Student's *t* test to compare mean values. Ridit analysis was performed to measure the distribution of the number of hepatoma.

Effect of EGCG on the growth of and AFP secretion by PLC/PRF/5 cells Human hepatoma-derived PLC/PRF/5 cells (Alexander cells) were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 50 μg/ml kanamycin in an atmosphere containing 5% CO_2 at 37°C. Four × 10^4 cells/ml were plated in Falcon 3013 flasks (25 cm^2) and 24 h later, the medium was changed to that containing 0, 0.1, 0.5, 1, 5, 10, 20, 40, 80, or 100 μ*M* EGCG. At day 6, total cell numbers were counted using Bürker-Türk plates and viabilities were determined by use of the trypan blue dye exclusion test. The culture media at day 6 were centrifuged at 7,600*g* for 15 min and the AFP content of the supernatant was measured using a radioimmunoassay (AFP RIA kit, Novo Laboratories Inc., Tokyo).[17]

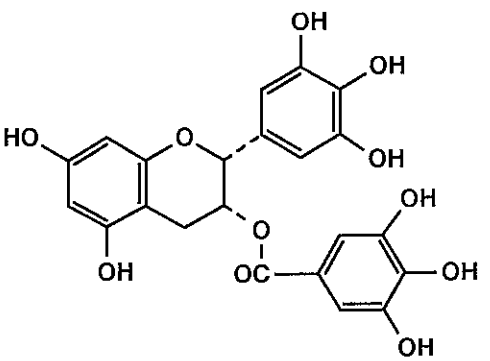

Fig. 1. Structure of (−)-epigallocatechin gallate (EGCG).

RESULTS

General observations There was no significant difference in the average body weight (Fig. 2), the average food consumption (data not shown) or the average water consumption (7.0 ml per day) among the three groups. The intakes of EGCG in group B and group C were estimated as 3.5 mg and 7 mg per day, respectively. During the experimental period, 2 mice in group A, 1 mouse in group B, and 3 mice in group C died.

Effect of EGCG on the development of hepatoma in C3H/HeNCrj mice EGCG significantly reduced the in-

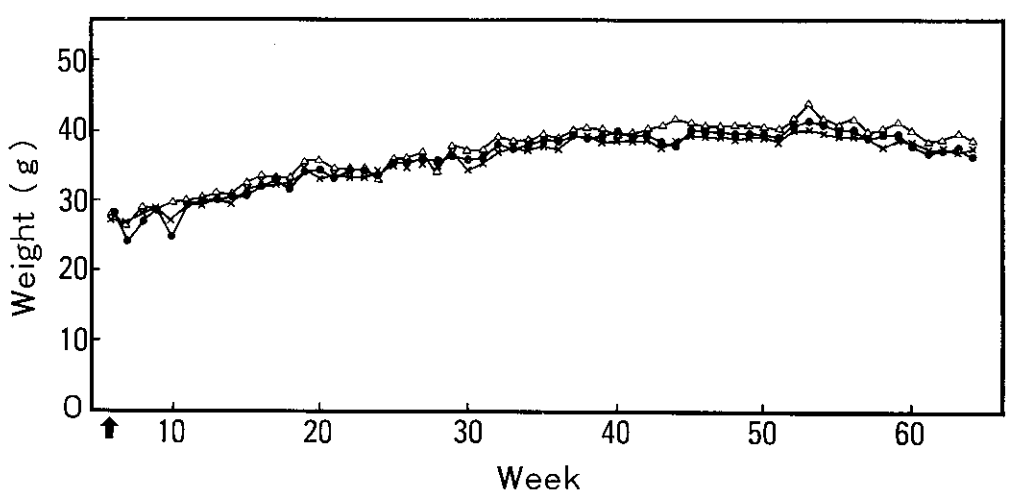

Fig. 2. Growth curves of mice in group A (without EGCG, △), group B (0.05% EGCG, ×) and group C (0.1% EGCG, ●). The arrow marks the beginning of EGCG administration. Administration of EGCG had no effect on the body weight.

cidences of hepatoma-bearing mice in group B (56.0%) and group C (52.2%) as compared to group A (83.3%) at week 58 of the experiment (Table I) ($P<0.05$). The average number of hepatomas per mouse was also significantly lower in group B (0.72 ± 0.74) when compared to group A (1.83 ± 2.28) ($P<0.05$). Groups A and C, however, did not significantly differ in the numbers of hepatomas per mouse (Table I). Table II stratifies the number of hepatomas per mouse into three categories: none, one or two, and three or more tumors. Ridit analysis revealed that the number of hepatoma-free mice in groups B and C was significantly increased as compared to the control ($P<0.05$). The values of average diameter of the hepatoma were 4.7 ± 4.9, 4.6 ± 3.1 and 5.6 ± 4.1 mm in groups A, B and C, respectively, and did not differ significantly. Microscopic observation of the tumors revealed that the proportions of hepatocellular adenoma and hepatocellular carcinoma in group A were 81.8% and 18.2%, those in group B were 83.3% and 16.7% and those in group C were 85.7% and 14.3%, respectively. There was no significant difference in the ratio among the three groups. These results indicated that EGCG treatment does not affect the histology of the tumors.

Effects of EGCG on cell growth and AFP secretion EGCG inhibited the growth of PLC/PRF/5 cells dose-dependently over the concentration range of 0.5 μM to 100 μM (Fig. 3). The viability of the cells exceeded 95% even with 100 μM EGCG, indicating that EGCG has low cytotoxicity. AFP secretion was reduced to approximately 60% of the control by treatment with the optimal concentration of EGCG (0.5 μM, Fig. 3). The amount of AFP secreted by 10^5 cells was 5.2 ng without EGCG and was 3.0 ng with 0.5 μM EGCG.

Analysis of blood serum No abnormality of any biochemical parameter was noted on analysis of blood in any of the three groups (data not shown), indicating that EGCG had no toxic effect on liver function.

DISCUSSION

In the present study, we demonstrated that EGCG, a major polyphenolic component of Japanese green tea, inhibits spontaneous hepatocarcinogenesis in C3H/HeNCrj mice. The bioavailability of EGCG to the liver is suggested by the report that glucuronide-conjugated EGCG can be found in the urine (Z. Y. Wang *et al.*, personal communication). Thus, the liver has been proven to be a target organ, in addition to the skin[11, 12] and duodenum,[13] of the anticarcinogenic activity of EGCG. It has also been shown that EGCG is effective not only at preventing chemical carcinogenesis[11–13] but also against spontaneously occurring tumors. Even though EGCG inhibited carcinogenesis significantly, no

Table I. Effects of EGCG on the Incidence and Average Number of Hepatomas in C3H/HeNCrj Mice

Group	No. of mice	Incidence (%)	Average number of hepatomas per mouse[a]
Without EGCG	18	15 (83.3)	1.83 ± 2.28
0.05% EGCG	25	14 (56.0)[b]	0.72 ± 0.74[b]
0.1% EGCG	23	12 (52.2)[b]	0.91 ± 1.08

a) Mean$\pm$SD.
b) $P<0.05$ as compared to the group without EGCG.

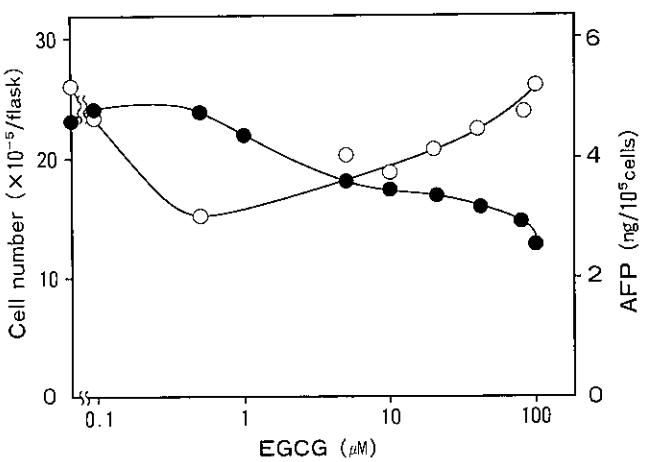

Fig. 3. *In vitro* effects of EGCG on the growth of PLC/PRF/5 cells (●) and the AFP level/10^5 cells in culture media (○).

Table II. Effects of EGCG on the Distribution of the Number of Hepatomas per Mouse

Group	No. of mice	Total numbers of hepatomas per mouse (%)		
		0	1–2	3–
Without EGCG	18	3 (16.7)	12 (66.7)	3 (16.7)
0.05% EGCG	25	11 (44.0)[a]	10 (40.0)	4 (16.0)
0.1% EGCG	23	11 (47.8)[a]	9 (39.1)	3 (13.0)

a) P(overall)<0.05 as compared to the group without EGCG.

toxicity in C3H/HeNCrj mice, in terms of blood biochemistry, growth, or food consumption, was evident throughout the experimental period, even in the group treated with as much as 0.1% EGCG.

However, the mechanisms by which EGCG exerts its anticarcinogenic effects are not yet clearly understood. Polyphenolic compounds, such as EGCG, are known to act as antioxidants. EGCG inhibits H_2O_2 formation induced by TPA in HeLa cells and suppressed the oxidation of lard as evaluated by the active oxygen method.[10, 18] These antioxidative activities of polyphenolic compounds are regarded as being associated with their antimutagenic activity in several experimental models.[19, 20] Since spontaneous hepatomas in C3H/HeNCrj mice are reported to have a high rate of c-Ha-*ras* oncogene mutations,[21] EGCG may exert its antimutagenic action by inhibiting activation of the c-Ha-*ras* gene in this mouse strain. In the present experiment, 10% (−)-epigallocatechin and 5% (−)-epicatechin gallate in addition to EGCG were also contained in the tea extract which was applied to the mice. Since these two additional catechins have comparable antioxidantive activities to EGCG,[10] it is likely that not only EGCG but also the two catechins exert anticarcinogenic activity in C3H/HeNCrj mice.

EGCG is also reported to have anti-tumor-promoting activity in two-stage carcinogenesis experiments in mouse skin. EGCG inhibited tumor promotion by two different types of tumor promoters, teleocidin A and okadaic acid,[22] and polyphenols are known to inhibit ornithine decarboxylase activity induced by tumor promoters.[23] Since EGCG inhibits the interaction of tumor promoters with their receptors, EGCG is believed to inhibit similarly the interaction of various growth factors and hormones with their receptors and, thereby, to inhibit specifically cell growth.[12, 24] Thus, it is conceivable that EGCG inhibits tumor promotion during hepatocarcinogenesis in C3H/HeNCrj mice, although no direct evidence is yet available.

The antitumorigenic effects of EGCG in C3H/HeNCrj mice have encouraged us to examine whether EGCG can inhibit the growth of human hepatoma-derived PLC/PRF/5 cells. EGCG dose-dependently inhibited the growth of PLC/PRF/5 cells without reducing their viability, while its ability to inhibit AFP production was dose-independent. AFP secretion was most strongly inhibited by as low a concentration as 0.5 μM EGCG — a concentration which caused minimal inhibition of cell growth. Maximum inhibition of cell growth was caused by EGCG at 100 μM, whereas AFP secretion/cell was not inhibited at that concentration (Fig. 3). The mode of action of EGCG may change depending on its concentration. At 0.5 μM, EGCG may induce differentiation and subsequently reduce AFP secretion by PLC/PRF/5 cells. We have not, however, studied the reciprocal increase of albumin secretion — a function of mature hepatocytes.[25]

The present study has revealed that EGCG inhibits both hepatocarcinogenesis in C3H/HeNCrj mice and the growth of PLC/PRF/5 cells without showing any toxicity. Since EGCG is widely consumed daily as green tea, it should not be difficult to allay concerns regarding its toxicity — the next step in our strategy for development of new cancer preventive agents.[26] Early clinical trials of EGCG should target those at high risk for hepatoma.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Hirota Fujiki, Saitama Cancer Center Research Institute, for valuable suggestions. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan and by a research grant from the Princess Takamatsu Cancer Research Fund.

(Received September 9, 1993/Accepted November 20, 1993)

REFERENCES

1) Oguni, I., Nasu, K., Kanaya, S., Ota, Y., Yamamoto, S. and Nomura, T. Epidemiological and experimental studies on the antitumor activity by green tea extracts. *Jpn. J. Nutr.*, **47**, 93–102 (1989).
2) Kono, S., Ikeda, M., Tokudome, S. and Kuratsune, M. A case-control study of gastric cancer and diet in northern Kyushu, Japan. *Jpn. J. Cancer Res.*, **79**, 1067–1074 (1988).
3) Ohno, Y., Aoki, K., Obata, K. and Morrison, A. S. Case-control study of urinary bladder cancer in metropolitan Nagoya. *Natl. Cancer Inst. Monogr.*, **69**, 229–234 (1985).
4) Wang, Z. Y., Khan, W. A., Bickers, D. R. and Mukhtar, H. Protection against polycyclic aromatic hydrocarbon-induced skin tumor initiation in mice by green tea polyphenols. *Carcinogenesis*, **10**, 411–415 (1989).
5) Wang, Z. Y., Agarwal, R., Bickers, D. R. and Mukhtar, H. Protection against ultraviolet B radiation-induced photocarcinogenesis in hairless mice by green tea polyphenols. *Carcinogenesis*, **12**, 1527–1530 (1991).
6) Wang, Z. Y., Huang, M.-T., Ferraro, T., Wong, C.-Q., Lou, Y.-R., Reuhl, K., Iatropoulos, M., Yang, C. S. and Conney, A. H. Inhibitory effect of green tea in the drinking water on tumorigenesis by ultraviolet and 12-*O*-tetradecanoylphenol-13-acetate in the skin of SKH-1 mice. *Cancer Res.*, **52**, 1162–1170 (1992).
7) Wang, Z. Y., Hong, J.-Y., Huang, M.-T., Reuhl, K. R., Conney, A. H. and Yang, C. S. Inhibition of *N*-nitro-

sodiethylamine- and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorigenesis in A/J mice by green tea and black tea. *Cancer Res.*, **52**, 1943–1947 (1992).
8) Yong, X. and Chi, H. The effect of Chinese tea on the occurrence of esophageal tumors induced by *N*-nitrosomethylbenzylamine formed *in vivo*. *Biomed. Environ. Sci.*, **3**, 406–412 (1990).
9) Rao, C. V., Tokumo, K., Rigotty, J., Zang, E., Kelloff, G. and Reddy, B. S. Chemoprevention of colon carcinogenesis by dietary administration of piroxicam, α-difluoromethylornithine, 16α-fluoro-5-androsten-17-one, and ellagic acid individually and in combination. *Cancer Res.*, **51**, 4528–4534 (1991).
10) Matsuzaki, T. and Hara, Y. Antioxidative activity of tea leaf catechins. *J. Agric. Chem. Soc. Jpn.*, **59**, 129–134 (1985).
11) Yoshizawa, S., Horiuchi, T., Fujiki, H., Yoshida, T., Okuda, T. and Sugimura, T. Antitumor promoting activity of (−)-epigallocatechin gallate, the main constituent of "Tannin" in green tea. *Phytother. Res.*, **1**, 44–47 (1987).
12) Yoshizawa, S., Horiuchi, T., Suganuma, M., Nishiwaki, S., Yatsunami, J., Okabe, S., Okuda, T., Muto, Y., Frenkel, K., Troll, W. and Fujiki, H. Penta-*O*-galloyl-β-D-glucose and (−)-epigallocatechin gallate: cancer preventive agents. *In* "Phenolic Compounds in Food and Their Effects on Health II," ed. M.-T. Huang, C.-T. Ho and C. Y. Lee, pp. 316–325 (1992). American Chemical Society, Washington.
13) Fujita, Y., Yamane, T., Tanaka, M., Kuwata, K., Okuzumi, J., Takahashi, T., Fujiki, H. and Okuda, T. Inhibitory effect (−)-epigallocatechin gallate on carcinogenesis with N-ethyl-N′-nitrosoguanidine in mouse duodenum. *Jpn. J. Cancer Res.*, **80**, 503–505 (1989).
14) Klaunig, J. E. Chemopreventive effects of green tea components on hepatic carcinogenesis. *Prev. Med.*, **21**, 510–519 (1992).
15) Yamauchi, O., Omori, M., Ninomiya, M., Okuno, M., Moriwaki, H., Suganuma, M., Fujiki, H. and Muto, Y. Inhibitory effect of sarcophytol A on development of spontaneous hepatomas in mice. *Jpn. J. Cancer Res.*, **82**, 1234–1238 (1991).
16) Frith, C. H. and Ward, J. M. A morphologic classification of proliferative and neoplastic hepatic lesions in mice. *J. Environ. Pathol. Toxicol.*, **3**, 329–351 (1985).
17) Fukutomi, Y., Omori, M., Muto, Y., Ninomiya, M., Okuno, M. and Moriwaki, H. Inhibitory effects of acyclic retinoid (polyprenoic acid) and its hydroxy derivative on cell growth and on secretion of α-fetoprotein in human hepatoma-derived cell line (PLC/PRF/5). *Jpn. J. Cancer Res.*, **81**, 1281–1285 (1990).
18) Bhimani, R. and Frenkel, K. Suppression of H_2O_2 production and oxidative DNA damage in HeLa cells by (−)-epigallocatechin gallate (EGCG). *Proc. Am. Assoc. Cancer Res.*, **32**, 126 (1991).
19) Mukhtar, H., Wang, Z. Y., Katiyar, S. K. and Agarwal, R. Tea components: antimutagenic and anticarcinogenic effects. *Prev. Med.*, **21**, 351–360 (1992).
20) Hayatsu, H., Inada, N., Kakutani, T., Arimoto, S., Negishi, T., Mori, K., Okuda, T. and Sakata, I. Suppression of genotoxicity of carcinogens by (−)-epigallocatechin gallate. *Prev. Med.*, **21**, 370–376 (1992).
21) Buchmann, A., Bauer-Hofmann, R., Mahr, J., Drinkwater, N. R., Luz, A. and Schwarz, M. Mutational activation of the c-Ha-*ras* gene in liver tumors of different rodent strains: Correlation with susceptibility to hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA*, **88**, 911–915 (1991).
22) Fujiki, H., Suganuma, M., Yoshizawa, S., Yatsunami, J., Nishiwaki, S., Furuya, H., Okabe, S., Nishiwaki-Matsushima, R., Matsunaga, S., Muto, Y., Okuda, T. and Suganuma, T. Sarcophytol A and (−)-epigallocatechin gallate (EGCG), nontoxic inhibitors of cancer development. *In* "Cancer Chemoprevention," ed. L. Wattenberg, M. Lipkin, C. W. Boone and G. J. Kellof, pp. 393–405 (1992). CRC Press, Boca Raton.
23) Gali, H. U., Perchellet, E. M. and Perchellet, J.-P. Inhibition of tumor promoter-induced ornithine decarboxylase activity by tannic acid and other polyphenols in mouse epidermis *in vivo*. *Cancer Res.*, **51**, 2820–2825 (1991).
24) Komori, A., Yatsunami, J., Okabe, S., Abe, S., Hara, K., Suganuma, M., Kim, S.-J. and Fujiki, H. Anticarcinogenic activity of green tea polyphenols. *Jpn. J. Clin. Oncol.*, **23**, 186–190 (1993).
25) Hirohashi, S., Shimosato, Y., Kameya, T., Koide, T., Mukojima, T., Taguchi, Y. and Kageyama, K. Production of α-fetoprotein and normal serum proteins by xenotransplanted human hepatomas in relation to their growth and morphology. *Cancer Res.*, **39**, 1819–1828 (1979).
26) Muto, Y., Ninomiya, M. and Fujiki, H. Present status of research on cancer chemoprevention in Japan. *Jpn. J. Clin. Oncol.*, **20**, 219–224 (1990).