RESEARCH ARTICLE

Anti-Tumor and Immunoregulatory Effects of Fermented Papaya Preparation (FPP: SAIDO-PS501)

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Abstract

Various beneficial effects have been described for fermented papaya preparation (FPP: SAIDO-PS501) based on its anti-oxidative and anti-inflammatory functions. The present study was designed to determine the effects of FPP on carcinogenesis *in vivo*, and immunomodulatory function *in vitro*. Mice were injected with RL male 1 cells subcutaneously or 3-methylcholantherene (MCA) intravenously to induce cancer and orally or intraperitoneally treated with FPP solution. Human peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers and patients with atopic dermatitis, treated with FPP, and subjected to measurement of cytokine production and changes in Foxp3-expressing regulatory T cell (Treg) stimulated with phytohemagglutinin (PHA). Administration of FPP suppressed tumor size and the incidence of malignancy. *In vitro*, treatment of PBMC with FPP induced IL-1β, TNFα and IFNγ production. Moreover, FPP suppressed proliferation of PHA-stimulated Foxp3-expressing Treg. These results suggest that FPP has chemotherapeutic properties.

Keywords: Fermented papaya preparation - anti-tumorigenesis - immunoregulatory function - interferonγ - regulatory

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Introduction

Fermented papaya preparation (FPP, SAIDO-PS501) is a natural functional food produced by fermentation of whole unripe Carica papaya Linn involving a variety of enzymes, and is marketed and used in Japan. Carica papaya Linn. is widely known as a traditional medicinal fruit with various properties such as antioxidant activity (Osato et al., 1993; Mehdipour et al., 2006), anti-tumor activity (Otsuki et al., 2010), promoter of immunity (Mohamed, 2012), wound healing effect (Nayak et al., 2012) and enhancement of phase II enzyme activity (Nakamura et al., 2000). In addition, Carica papaya Linn. contains various beneficial compounds, such as α-tocopherol (Ching and Mohamed, 2001), flavonoid (Miean and Mohamed, 2001), benzylisothiocyanate (Basu and Haldar, 2008) and lycopene (van Breemen and Pajkovic, 2008) with anti-cancer properties. We reported previously that FPP exhibits free radicals-scavenging activity and has beneficial effects against certain diseases in which oxidative stress (OS) plays a pathological role (Imao et al., 1998; Noda et al., 2008). Moreover, FPP provides protection against intestinal allergy through its anti-inflammatory and immunoregulatory properties (Hiramoto et al., 2008). However, the precise mechanisms of these actions are still unknown.

The incidence and mortality rates of cancer have increased in recent years (Kamangar et al., 2006). Although various treatment modalities are available for cancer, such as surgery, chemotherapy, and radiotherapy, more optimal therapy and prevention strategies have yet to be established. The etiology of cancer is multifactorial, including OS and chronic inflammation, which can cause DNA and tissue damage (Murata et al., 2012). Recent evidence indicates that consumption of vegetables and fruits increases antioxidant activity and reduces the risk of various diseases including cancer. Indeed, some foods and beverages, such as tea, tomato, soybean, have high antioxidant activity and are effective in prevention of cancer (Kennedy, 1995; Basu and Imrhan, 2007; Chen and Zhang, 2007; Harasym and Oledzki, 2014).

On the other hand, cancer prevention depends mainly on anti-tumor immune functions through cellular immunity involving both innate and adaptive immunity (Vesely et al., 2011). Immunostimulation is considered helpful as a therapeutic intervention in cancer patients. Certain foods have recently been demonstrated to have immunostimulatory effect. For example, lentinan, which is (1-3)-beta-d-glucan purified from the Shiitake mushrooms, is reported to have immunomodulatory and anti-tumor effects through Toll-like receptors (TLR) (Taguchi, 1987; Nakano et al., 1999; Pamer, 2004; Gantner et al., 2003), similar to sizofiran (Tokuyasu et al., 2010). In addition, lipopolysaccharide (LPS) and peptidoglycan induce cytokine release via up-regulation of TLR4 and TLR2 on the surface of monocytes (Hadley et al., 2005). It is noteworthy that suppression of host anti-tumor immune mechanisms is partly due to the actions of CD4+

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and forkhead box p3 (Foxp3+)-expressing regulatory T cells (Treg). It has been reported that excess accumulation of Treg enhances cancer progression and/or reduces the effects of chemotherapy, suggesting that inhibition of Treg is a potentially useful therapeutic strategy against cancer (Onizuka S et al., 1999; Ohkusu-Tsukada K et al., 2010).

Thus, it is expected that antioxidants with both immunostimulatory activity and immunomodulatory properties hold promise as new innovative agents for cancer therapy. The present study was designed to investigate the anti-tumor effects of FPP in both RL male 1 and methylcholanthrene (MCA) mouse model, including elucidation of the mechanisms of action, with a special focus on the immunoregulatory function.

Materials and Methods

Animals

Female BALB/c mice (age, 6-8 weeks) were purchased from Japan SLC (Shizuoka, Japan) or bred in our laboratory animal center. The mice were housed into cages at random and provided standard mouse diet and water. The room was maintained at 23°C with 12/12-h light-dark cycle. All animal procedures were in strict accordance with the NIH Guideline for the Care and Use of Experimental Animals and the Guideline for Animal Experiments of Okayama University Advanced Science Research Center, and were approved by the Animal Care and Use Committee of Okayama University Advanced Science Research Center. Special care was taken to minimize the number of animals used in this research.

An experimental mouse model of cancer was prepared as described previously together with the slight modification outlined by our group (Andreesen R et al. 1998; Proietti E et al., 1993). Briefly, mice were anesthetized by inhalation of diethylether and then either inoculated with 2×10^5 cells/100 μl in phosphate buffered saline (PBS) obtained from RL male 1 BALB/c mice with radiation-induced leukemia, or injected subcutaneously with 3-methylcholantherene (MCA) 200 μg/100 μl in peanut oil.

FPP (SAIDO-PS501®) was kindly provided by SAIDO Corporation (Fukuoka, Japan). For oral administration, it was dissolved in tap water to concentrations of 180, 360, and 540 mg/200 ml (relative dose: 150, 300 or 450 mg/kg/day). These doses were equal to 3, 6 and 9 packages available in the market for human consumption (9, 18, and 27g/60 kg/day)). For intraperitoneal injection, FPP was dissolved in saline to final concentrations of 40 and 100 mg/250 μl (relative dose: 1600 or 4000 mg/kg/2 days). The longitudinal and horizontal axes of the tumor were measured weakly and their mean values were recorded and expressed as tumor size.

Human peripheral blood mononuclear cells

Heparinized peripheral blood samples were obtained from healthy donors (HD) and patients with atopic dermatitis (AD) who were being treated at that of the study with immunosuppressants (topical steroids) after obtaining informed consent. Human peripheral blood mononuclear cells (PBMC) were prepared by the Histo-Paque technique (Sigma Aldrich, St. Louis, MO). These samples were obtained before and after 2-month intake of 3 g FPP. For in vitro treatment of PBMC, FPP was dissolved in PBS and incubated at 37°C for 1 h. The solution was centrifuged at 2000 rpm for 10 min, and the supernatant was filtered using 0.45 μm filter. The prepared PBMC were treated with FPP at final concentration of 10 mg/ml. In addition to the vehicle, glucose (Katayama Chem. Japan) was used as control, because glucose is abundant in FPP. PBMC were stimulated with LPS derived from Escherichia coli (Sigma Aldrich, St. Louis, MO) dissolved in PBS to a final concentration of 1 μg/ml.

Separation of CD8+ and CD4+ T cells

T cell separation was carried out by magnet sorting with anti-CD8 or CD4 antibody conjugated to iron beads (Miltenyi Biotech, Germany) according to the instructions provided by the manufacturer. Whole human PBMC, CD8+ and CD4+ cells were stimulated with phytohemagglutinin (PHA) for 11 days in the presence of medium only, FPP or glucose. The cell number was counted by microscopy and the concentration of IFNγ in the culture medium was measured by enzyme-linked immunosorbent assay (ELISA).

Cytokine assay

PBMC were plated onto a 96-well microplate at a concentration of 1×10^5 or 1×10^6 cells/200 μl/well in the presence of FPP and/or LPS or glucose for 24 h at 37°C in humidified incubator with 5% CO_2. TLR4-expressing human pro-monocyte cell line, THP-1, was cultured in RPMI medium with 10% fetal calf serum (FCS). The levels of interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) in the supernatants were measured by ELISA kit (R&D Systems, Minneapolis, MN) using the instructions provided by the manufacturer. The level of interferon gamma (IFNγ) was determined by our laboratory procedure using the sandwich ELISA method. In this method, 96-well plate pre-coated with mouse anti-human INFγ (1:500; Mabtech, Mabtech AB) in carbonate buffer (pH 9.6) as a capture antibody, were blocked with 5% fetal calf serum (FCS) in PBS for 2 h at 37°C, followed by washing with 0.05% Tween 20 in PBS. The supernatant of the incubated cell medium after treatment was transferred to the plates and plated for 1 h, and then reacted with rabbit anti-human IFNγ antibody, which was produced by immunization with pure human IFNγ (Calbiotec, CA) in our laboratory, in 5% FCS/PBS for 1 h at 37°C. After washing, goat anti-rabbit-IgG-HRP (dilution, 1:2000; MBL) in 5% FCS/PBS was added to the wells and the plates were incubated for 1 h at 37°C. For detection, OPDA (Wako, Japan) was dissolved at 3 mg/10 ml in citrate buffer (pH 5.0) with 10 μl of 31% H_2O_2. Absorbance was determined at optical density of 490 nm using a microplate reader (Bio-Rad, Hercules, CA).

Flow cytometry

The following antibodies were used: anti-hCD4-allophycocyanin (eBioscience, San Diego, CA), anti-hCD8-allophycocyanin (eBioscience), and anti–hFoxp3-PE (eBioscience) antibodies. Intracellular Foxp3

staining was performed using a Foxp3 staining buffer set (eBioscience) according to the instructions supplied by the manufacturer. To detect, CD4 and CD8 T cells, PBMC were stained with anti-hCD4-allophycocyanin or anti-hCD8-allophycocyanin antibody, respectively. Measurement was performed with FACS Calibur and analysis was conducted using the Cell Quest software (Beckman Coulter, Fullerton, CA).

Limulus test

The LPS titer of FPP was estimated by the Limulus test using a commercially available Limulus Amebocyte Lysate LAL Kinetic-QCL Kit (Cambrex Bio Science, MD), and performed according to the instructions provided by the manufacturer.

Reversed-phase high performance liquid chromatography

Analysis using reversed-phase high performance liquid chromatography (HPLC; model 151A, Applied Biosystem) was performed under the conditions of separation column; C18 (Vydac, cat. No 218), flow rate; 1 ml/min, and injection volume; FPP (400 mg/ml) 100 μl, LPS (0.4 mg/ml) 37 μl. These volumes were determined from the equivalent point of titration curve. The fractionated material was collected every 1 min and freeze-dried for neutralization, and resolved in 100 μl PBS to stimulate PBMC.

Statistical analysis

Data are expressed as mean ± SD (n=3). Differences between groups were examined for statistical significance using the two-tailed unpaired Welch's t-test or Mann-Whitney's U test, while differences among several groups were examined by one-way analysis of variance (ANOVA). A two-tailed P value less than 0.05 was considered statistically significant.

Results

Anti-tumor effect of FPP

Inoculation of RL male 1 (2×10^5 cells) resulted in tumor development with gradual increase in tumor size from days 4 to 6 (Figure 1A, C, E). Oral administration of FPP dose-dependently suppressed tumorigenesis in RL male 1 mice (Figure 1). At postinoculation day 18, oral administration of FPP at 150 mg/kg/day did not affect tumor size (Figure 1B), but the dose of 300 mg/kg/day decreased the tumor size by approximately 30% relative to the glucose group (Figure 1D), and a higher dose of 450 mg/kg/day resulted in complete resolution of the tumor (Figure 1F).

In another series of experiments, inoculation of RL male 1 (2×105 cells) was followed by the appearance and growth of subcutaneous tumor, with a transient decrease in tumor size noted between postinoculation days 12-18 (Figure 2A, C). Intraperitoneal administration of FPP (1600 or 4000 mg/kg/2 days) did not affect the development and progression of the tumor (Figure 2). At postinoculation day 18, the mean tumor size was similar in FPP- and glucose-treated mice (Figure 2B, D).

Injection of MCA (200 μg) resulted in the appearance of subcutaneous tumor, and the incidence of the tumor increased with time (Figure 3). Oral administration of FPP 300 mg/kg/day reduced tumor incidence in MCA-injected mice (Figure 3A). The tumor incidence was spontaneously lower at postinoculation week 18 in water- and glucose-treated mice (87% and 85%, respectively). However, oral administration of FPP further diminished the incidence at the same period to 55% (Figure 3B), and the incidence was 63% of that of the water group. This experiment was repeated twice and the same results were obtained with a suppression rate of 67% (data not shown).

FPP stimulates PBMC and modulates cytokine production in vitro

To investigate the mechanisms of the anti-tumor effect of FPP in mice, we examined its effect on cytokine production by PBMC obtained from HD and AD. For this

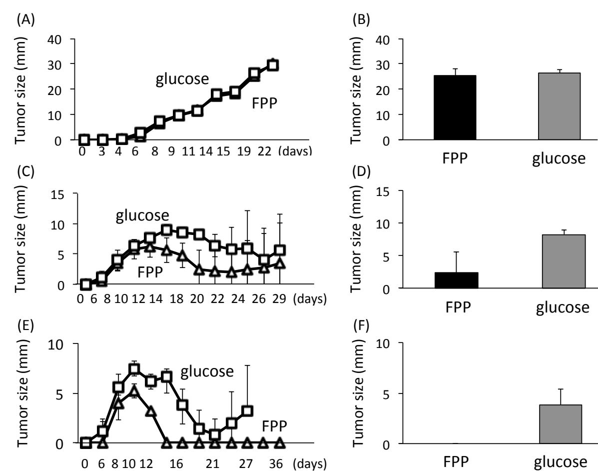

Figure 1. Dose-dependent Effects of Orally Administered FPP on Tumorigenesis Induced by RL Male 1. (A, C, E) Time-course changes in tumor size and (B, D, F) mean tumor size at 18 days in mice administrated 150 mg/kg/day (A, B), 300 mg/kg (C, D), and 450 mg/kg (E, F) FPP or glucose. RL male 1 injection: 2×10^5 cells/100 μl. Data are mean±SEM (n=5)

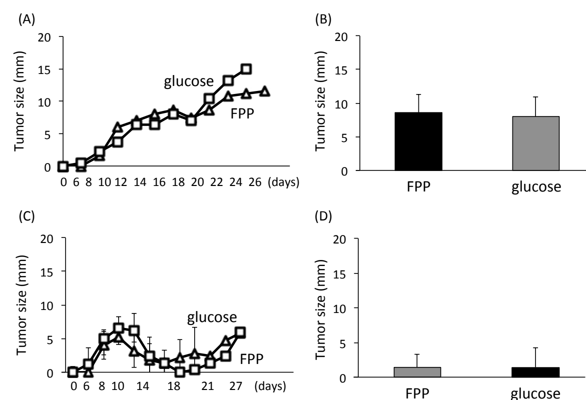

Figure 2. Effects of Intraperitoneal Administration of FPP on Tumorigenesis Induced by RL Male 1. (A, C) Time-course changes in tumor size and (B, D) mean tumor size at 18 days in mice injected intraperitoneally 1600 mg/kg/2 days (A, B), and 4000 mg/kg/2 days (C, D) FPP or glucose. RL male 1 injection: 2×10^5 cells/100 μl. Data are mean±SEM (n=5)

Table 1. Effect of FPP on T cell proliferation. Proliferation of CD8+ and CD4+ T cell at 11 days after stimulation with 10 μg/ml PHA was determined by microscopy. Starting cell number: 1 × 10⁵ cells/5 cm well plate

Cell type	Naive	FPP (10 mg/ml)	glucose (10 mg/ml)
Total PBMC	2.4 x 10^6	1.2 x 10^6	2.7 x 10^6
CD8+ T cells	1.2 x 10^6	1.5 x 10^6	2.2 x 10^6
CD4+ T cells	11.1 x 10^6	7.6 x 10^6	9.0 x 10^6

PHA stimulation: 10 μg/ml for 11 days; Starting cell count: 2 x 10^5 cells /5 cm-well plate

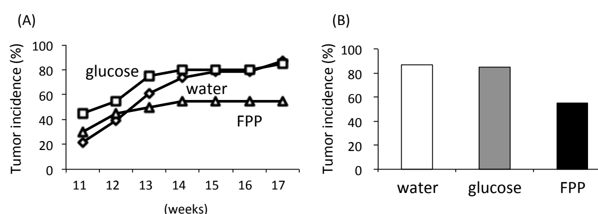

Figure 3. Effects of Oral Administration of FPP on Tumorigenesis Induced by MCA. (A) Tumor incidence at 11-17 weeks and (B) at 18 weeks after injection of 200 μg MCA in mice. FPP and glucose were administered at a dose of 300 mg/kg/day. Data are mean values (n=20)

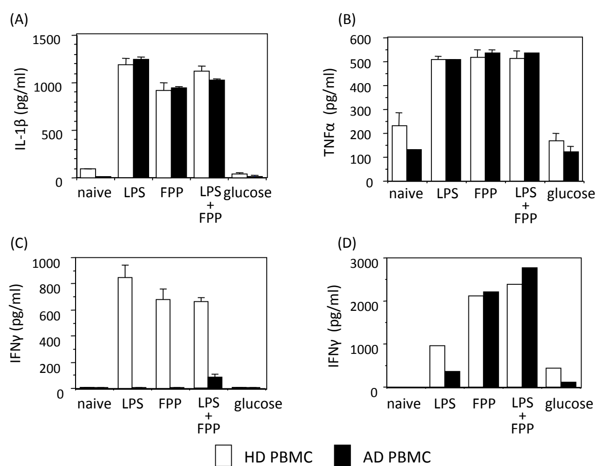

Figure 4. Effects of FPP on Cytokine Production by Peripheral Blood Mononuclear Cells (PBMC) from Healthy Donors (HD) and Patients with Atopic Dermatitis (AD). Effects of glucose, LPL, or FPP on (A) IL-1β, (B) TNFα, (C) IFNγ (at 0 month), and (D) IFNγ (at 2 months) production from PBMC obtained from HD and AD. Oral FPP 3 g/day, LPS (1 ng/ml) with/without FPP or glucose (10 mg/ml) for 24 h. Data are mean±SD

purpose, we measured cytokine levels in cultured media of PBMC obtained from HD and AD by ELISA. Culture of cells obtained from untreated HD and AD subjects in the presence of FPP 10 mg/ml or/and LPS 1 ng/ml or glucose 10 mg/ml for 24 h induced IL-1β (Figure 4A) and TNFα (Figure 4B), whereas FPP or/and LPS induced IFNγ production by PBMC from HD but not AD (Figure 4C). After 2 months of treatment of HD and AD with FPP at 3 g/day, stimulation of PBMC obtained from the treated subjects with FPP or/and LPS induced comparable

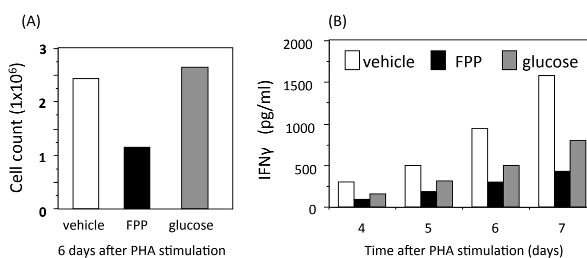

Figure 5. Effect of FPP on PHA-stimulated PBMC. Effects of exposure of PBMC pre-treated with 10 mg/ml FPP and glucose for 24 h to 10 μg/ml PHA on (A) cell count at 6 days and (B) IFNγ production. Data are mean ± SD

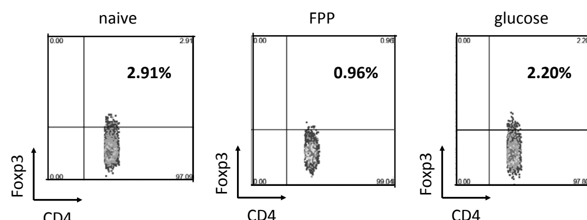

Figure 6. Effect of FPP on Non-Specific Proliferation of Regulatory T Cells. PBMC were treated with 10 μg/ml PHA for 11 days and changes in CD4 and Foxp3 expression were analyzed by FACS analysis. The effect of 10 mg/ml FPP and glucose was examined separately by incubation of pretreated PBMC for 24 h

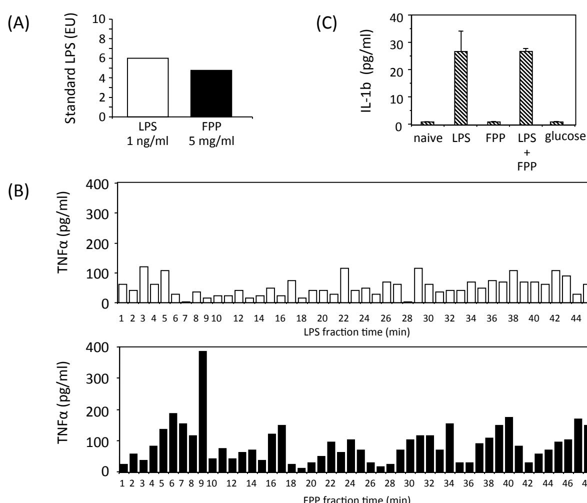

Figure 7. Differences in Adjuvanticity Between LPS and FPP. (A) Results of Limulus test for LPS and FPP. (B) TNFα production level by PBMC treated with HPLC fractions (collected per 1 min) of LPS and FPP. (C) IL-1β production. Promonocyte cell line THP-1 respond to LPS, but not to FPP or glucose. Data are mean±SD (n=3)

IFNγ production levels by PBMC from both AD and HD (Figure 4D).

FPP suppresses PHA-induced immunoresponse

PHA (10 μg/ml), a non-specific stimulator of T cells, induced cell proliferation and IFNγ production by PBMC pre-treated with FPP and glucose (Figure 5). Quantitative analysis showed higher T cell count

at post-PHA stimulation day 6, relative to water- and glucose-incubated PBMC (Figure 5A). In addition, PHA stimulation increased IFNγ production by PBMC in a time-dependent manner (Figure 5B). These changes were abrogated by incubation of the cells with 10 mg/ml FPP.

Effect of FPP on PHA-stimulated Treg proliferation

Further examination showed that after 11-day PHA stimulation, the total PBMC cell count increased to 2.4×10^6 cells, CD8+ T cells to 1.2×10^6 cells, and CD4+ T cells to 11.1×10^6 cells, among naive PBMC (Table 1). For PBMC pre-treated for 24 h with FPP 10 mg/ml, the number of total PBMC was reduced to 1.2×10^6 cells, reflecting reduction of the numbers of CD4+ T cell (7.6×106 cells), but not CD8+ T cells (1.5×10^6 cells). Treatment with glucose did not affect the proliferation of PHA-stimulated PBMC.

FPP suppresses PHA-stimulated Treg expression

Foxp3 is the most specific marker of Treg. We analyzed the induction of Foxp3 expression in CD4+ cells extracted from PHA-stimulated PBMC by FACS analysis. As shown in Figure 6, Foxp3+/CD4+ T cells constituted 2.91 and 2.20% of the total CD4 cells among naive and glucose-treated PBMC in response to PHA stimulation, respectively. On the other hand, FPP treatment reduced the ratio of Foxp3+/CD4+ T cells to 0.96%, suggesting that FPP suppressed PHA-induced Treg proliferation.

Differences in adjuvanticity between FPP and LPS

To rule out possible contamination of FPP actions with LPS, we used the Limulus test to examine differences in the capacity of FPP and LPS in inducing cytokine production from PBMC, using reverse-phase HPLC. The Limulus test showed that the potency of 1 ng LPS was equivalent to 5 mg FPP (Figure 7A). Furthermore, the peak TNFα production appeared at 8-9 min in the FPP HPLC fraction, but not in the LPS fraction (Figure 7B). Similar results were obtained for IL-6 secretion (data not shown). In addition, while the immunostimulatory effect of LPS was inactivated by treatment with acetonitrile and trifluoroacetic acid, these two compounds had no effect on FPP (data not shown). Moreover, LPS, but not FPP, increased TNFα production from TLR4-positive THP-1 cells (Figure 7C).

Discussion

There is no doubt a need for new ideal preventive therapeutic strategies against cancer. We report here the anti-tumor effect of FPP in vivo in mouse cancer models. First, Mice inoculated with BALB/c mouse leukemia RL male 1 developed subcutaneous tumors. Oral administration of FPP inhibited tumor growth in these mice in a dose-dependent manner. Moreover, oral administration of FPP at dose of 450 mg/kg/day results in complete disappearance of the tumor. Interestingly, these effects were not observed after intraperitoneal administration of FPP even when the latter was used at higher doses. Second, we also examined the effects of oral FPP administration against MCA-induced carcinogenesis. The incidence of carcinogenesis following MCA injection was 87% and 85% in the water- and glucose-treated mice, respectively, but it was lower (55%, i.e., 63% suppression) following oral treatment with FPP.

In general, cancer prevention depends mainly on immune cells, such as monocytes, macrophages and natural killer (NK) cells. It has been reported that these cells act on cancer prevention while their absence increases cancer risk (Carswell et al., 1975). Thus, the anti-tumor immune system is considered a potential target for cancer therapy. To elucidate the mechanisms of the anti-tumor effect of FPP, we investigated whether FPP can activate immune function in vitro. IL-1β, TNFα and IFNγ released by immunocytes are involved in numerous immunological and inflammatory reactions and can inhibit tumor progression (Esumi et al., 1991; Harutsumi et al., 1995; Li et al., 2014). IFNγ can also enhance macrophage and NK cell activity. In the present study, treatment with FPP stimulated IL-1β, TNFα and IFNγ production from PBMC of HD in vitro, suggesting that FPP can activates innate immunity. In PBMC obtained from AD, neither FPP nor LPS treatment increased IFNγ production, but not that of IL-1β and TNFα. Atopic or allergic patients have Th2 cytokine pattern and lower IFNγ levels are involved in defective innate immune system (Katagiri et al., 1997; Hino et al., 2005). In the present study, daily intake of FPP for 2 months resulted in recovery in the ability of PBMC of AD to produce IFNγ in response to FPP or/and LPS, to levels comparable to those observed in HD. These results suggest that FPP can potentiate a change from Th2 to Th1 type and increase innate immunity through both direct and indirect pathways. Our results also suggest that FPP can improve atopic or allergy syndrome and exert anti-tumor effects via augment of innate immunity by increasing cytokine production from PBMC.

Recently, immunotherapeutic strategies against cancer have focused on not only enhancement of immunity by immunocytes, but also canceling immunosuppression against cancer. Previous reports suggested that Treg inhibit anti-tumor immunity, and suppression of Treg is a candidate new cancer preventive strategy (Onizuka et al., 1999; Ohkusu et al., 2010). In the present study, stimulation of PBMC by PHA resulted in non-specific cell proliferation, IFNγ over-production and increased proportion of Foxp3+/CD4+ Treg, while treatment with FPP abrogated these changes. Taken together, the above results suggest that FPP exhibits unique immunoregulatory effects through enhancement of the immune system and regulation of excess immunoreactions and anti-tumor immunity.

LPS is a cell-wall component of Gram-negative bacteria and has immunostimulatory activity. LPS can prevent cancer via TLR4 signaling (Li et al., 2014; Reinhold U et al., 1990), however, excess LPS acts as an endotoxin and can damage or impair tissue function. In the present study, FPP treatment induced IL-1β, TNFα and IFNγ secretion from PBMC, similar to LPS. We used the Limulus test to check possible contamination of FPP actions by LPS. The test showed that LPS level in 5 mg of FPP was equivalent to that in 1 ng LPS, suggesting that FPP includes a small amount of LPS. However,

the same test is also known to react with β-D-glucan, in addition to LPS (Sandle, 2013). We also showed the ability of the FPP fraction, but not that of LPS, to stimulate TNFα and IL-6 production. Moreover, LPS, but not FPP, increased IL-1β production by THP-1, TLR4-expressing human promonocyte cell line. These results indicate that the immunostimulatory and anti-tumor activities demonstrated in the present study are mainly due to FPP rather than to LPS present in small amount in FPP.

Previous studies demonstrated that FPP prepared from Carica papaya Linn. has nutritional and functional properties, including antioxidant, anti-inflammatory, immunostimulating and anti-tumor activities (Osato et al., 1993; Mehdipour et al., 2006; Krishna et al., 2008; Otsuki et al., 2010; Mohamed, 2012; Nguyen et al., 2013; Sagnia et al., 2014). FPP also protects against certain OS-related diseases, such as traumatic epilepsy (Imao et al., 1998), allergic inflammations (Hiramoto et al., 2008), scopolamine-induced amnesia (Imao et al., 2001), Alzheimer's disease (Zhang et al., 2006), stress-induced acute gastric mucosal lesion (Murakami et al., 2012), and non-alcoholic steatohepatitis (Murakami et al., 2013), in experimental cell and animal models. Several studies also reported the anti-tumor effects of antioxidants. For example, N-acetylcysteine influences redox balance and modulates immune system via cytokines production from PBMC (Al-Shukaili et al., 2009; Mantovani et al., 2000; De la Fuente and Victor, 2000). In our latest study, we also showed that dietary intake of FPP can dose-dependently activate NK cell cytotoxicity in the elderly and as well as modulate intestinal microflora (submitted manuscript). Gut microflora play an important role in immune function, and recent studies reported the possible involvement of the gut microbiota in immune response against various diseases including cancer (Iida et al., 2013; Viaud et al., 2013). It has been demonstrated that some prebiotics and probiotics, including yoghurt, have anti-tumor activity through their immunostimulatory and immunoregulatory properties (Rafter, 2003; de Moreno de Leblanc and Perdigon, 2004; van Hemert et al., 2010). These properties may be attributed to improvement of the microflora environment (Zhong et al., 2014). The present study showed that the anti-tumor effects of FPP were only noted after oral but not intraperitoneal administration, suggesting that these effects are most likely mediated through alteration in microflora-related digestion and/or metabolism.

The present study has certain limitations. We cannot identify the active FPP component(s) responsible for the observed anti-tumor effects, because it is too difficult to isolate and identify the active ingredients because FPP contains a large number of nutritional components. Anti-tumor components, such as glutamic acid, cystine, cytosine, nicotinic acid, pipecolic acid, homoserine, quinic acid and glucuronic acid, including FPP were identified by metabolome analysis, however, they have not been quantified yet (data not shown). Consumption of nutritional antioxidants is known to be effective in reducing the risk of cancer, not only based on the antioxidant activity of individual components, but also probably on synergistic interaction with not yet known antioxidants or synergy of these components. We also consider that the multifunctional properties of FPP are attributed to various interactions between their components. Together with previous study and the present results, we believe that the anti-tumor effects of FPP are likely related not only to direct action, but also indirect action including modulation of digestion, metabolism and microflora associated with immunoregulatory activity. Further studies are necessary to elucidate the mechanisms of action and to demonstrate the clinical efficacy in cancer patients.

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100.0
75.0
50.0
25.0
0
6.3
56.3
31.3
Newly diagnosed without treatment

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