

# Purple corn color inhibition of prostate carcinogenesis by targeting cell growth pathways

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Purple corn color is a widely used food colorant that was reported to have attenuating effects on hypertension, diabetes, and to have anti-cancer effects on colon and breast cancer. Our study is the first on its possible chemoprevention effects against prostate cancer. For this purpose an androgen-dependent prostate cancer cell line, LNCaP, was used to examine effects *in vitro*. Purple corn color inhibited the proliferation of LNCaP cells by decreasing the expression of Cyclin D1 and inhibiting the G1 stage of the cell cycle. Thirty-six male transgenic rats for adenocarcinoma of prostate were fed basic diet or diet with purple corn color for 8 weeks. Purple corn color decreased the incidence of adenocarcinoma in the lateral prostate and slowed down the progression of prostate cancer. A lower Ki67 positive rate, a decrease of the expression of Cyclin D1, and downregulation of the activation of Erk1/2 and p38 MAPK were observed in the group consuming purple corn color in the diet. Since purple corn color is a mixture, determining its active component should help in the understanding and usage of purple corn color for prostate cancer chemoprevention. Therefore, the three major anthocyanins in purple corn color, cyanidin-3-glucoside, pelargonidin-3-glucoside and peonidin-3-glucoside, were tested with LNCaP cells. The results suggested that cyanidin-3-glucoside and pelargonidin-3-glucoside are the active compounds. (*Cancer Sci* 2013; 104: 298–303)

Cancer is a major public health problem in developed and some developing countries. In 2012, prostate cancer (PCa) was estimated to be the No.1 cause of new cancer cases in males and the second highest cause of cancer related deaths in the USA,<sup>(1)</sup> and PCa is rapidly increasing in Asia. Although considerable effort has been expended on searching for early screening markers and curing PCa, the main treatment remains androgen ablation therapy, which was developed in the early 1940s.<sup>(2,3)</sup> However, even though more than 80% of PCa respond to this therapy, almost all of these cases relapse in less than a decade and become refractory to treatment.<sup>(3)</sup> Brachytherapy, radiotherapy, and prostatectomy of PCa prior to metastasis can affect a cure, but these procedures can dramatically alter the quality-of-life of the patient.<sup>(4,5)</sup> Therefore, prevention of PCa is especially important.

The field of chemoprevention, using natural or laboratory-made substances to prevent cancer, has become increasingly studied in recent years. Researchers have investigated numerous chemicals that may have chemopreventive effects on PCa *in vitro* and *in vivo*. Three large-scale randomized, controlled clinical trials have been conducted: the SELECT trial found that neither selenium nor Vitamin E reduced the risk of PCa in healthy men at average risk;<sup>(6)</sup> the PCPT trial found that finasteride, a 5 $\alpha$ -reductase inhibitor, reduced the risk of PCa of Gleason 6 or less, whereas there was an increased risk of high grade disease with Gleason 7 or more;<sup>(7)</sup> the REDUCE

trial also encountered similar difficulties with dutasteride, another 5  $\alpha$ -reductase inhibitor.<sup>(8)</sup> Therefore, the search for an appropriate chemopreventor for PCa needs to be continued.

Purple corn has a long history as a food product. Nowadays its color is widely used as a food colorant in Japan. Previous studies have provided evidence that purple corn color (PCC) has anti-cancer effects on colon and breast cancer.<sup>(9,10)</sup> It also has attenuating effects on some life style diseases, for example, hypertension, hyperglycemia, and diabetes.<sup>(11,12)</sup> The present study was conducted as an initial investigation on PCC's effects on PCa. As a result we found that PCC inhibited the proliferation of the androgen-dependent cell line LNCaP *in vitro* and inhibited prostate carcinogenesis *in vivo* in the Transgenic Rat for Adenocarcinoma of Prostate (TRAP) model. The TRAP rat model, in which expression of the Simian virus 40 T antigen is under control of the probasin gene promoter, was established in our laboratory. These animals develop high-grade prostatic intraepithelial neoplasia (HG-PIN) and well-differentiated adenocarcinomas with high incidence in all prostate lobes at 15 weeks of age, all lesions being completely androgen-dependent.<sup>(13,14)</sup> The model provides an ideal tool to gain insights into possible mechanisms of PCa prevention in relatively short-term studies.<sup>(15–19)</sup>

Purple corn color is a mixture, which contains several anthocyanins. To determine its active component, the three major anthocyanins in PCC, cyanidin-3-glucoside (C3G), pelargonidin-3-glucoside (Pg3G) and peonidin-3-glucoside (P3G), were tested using LNCaP cells. By comparing the effects of these anthocyanins to the effect of the mixture, we found that C3G and Pg3G are the active compounds.

To our knowledge, the present study provides the first evidence that PCC inhibits prostate carcinogenesis in a rat model closely mimicking the human disease. The clues obtained as to the molecular basis of action are of critical importance as first steps towards human clinical trials.

## Materials and Methods

**Chemicals, reagents, plasmids and cell line.** Purple corn color was provided by San-Ei Gen F.F.I. (Osaka, Japan). Lot No. 100413, anthocyanin concentration 12.5% was used for the *in vitro* study. Lot No. 110418, anthocyanin concentration 20.9% was used for the *in vivo* study. C3G (Cyanidin-3-O-glucoside chloride) and P3G (Peonidin-3-O-glucoside chloride) were purchased from Tokiwa Phytochemical (Chiba, Japan). Pg3G (Pelargonidin-3-O-glucoside chloride) was purchased from Extrasynthese (Genay Cedex, France). The chemical structures of C3G, P3G and Pg3G are shown in Supplementary

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Figure S1. The LNCaP human PCa cell line (androgen-dependent) was from the American Type Culture Collection (Manassas, VA, USA). The pGL3-PSA luciferase expression vector (pGL3/PSA-Luc) was donated by Dr Chawnsang Chang, University of Rochester Medical Center.

**Animals.** Male heterozygous TRAP rats with a Sprague-Dawley genetic background were used in the present study. They housed three animals per cage on wood-chip bedding in an air-conditioned animal room at  $23 \pm 2^\circ\text{C}$  and  $50 \pm 10\%$  humidity. Food and tap water were available *ad libitum*. The Institutional Animal Care and Use Committees of the Nagoya City University (Nagoya, Japan) specifically approved this study.

**Experimental protocol.** A total of 36 heterozygous male TRAP rats at 6 weeks of age were randomly divided into three groups. Rats in the control group ( $n = 13$ ) received powdered basal diet (Oriental MF, Oriental Yeast, Tokyo, Japan). The rats in the other two groups received 0.1% ( $n = 12$ ) or 1% PCC ( $n = 11$ ) in the diet for 8 weeks. At the end of week 8, the rats were killed under deep anesthesia. Each prostate was removed and halves of the ventral and lateral lobes were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until processed; the remaining prostates was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. Testosterone and estrogen levels in the serum were analyzed by radioimmunoassay by SRL (Tokyo, Japan). All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences.

**Assessment of prostate neoplastic lesion development.** Neoplastic lesions in the prostate glands of TRAP rats were evaluated as previously described.<sup>(19)</sup> Briefly, neoplastic lesions were classified into three types: low-grade prostatic intraepithelial neoplasia (LG-PIN), HG-PIN and adenocarcinoma. The relative numbers of acini with the histological characteristics of each type, that is, LG-PIN, HG-PIN and adenocarcinoma, were quantified with reference to the total acini in each prostatic lobe.

**Immunoblot analysis.** The immunoblotting analysis was performed as described previously.<sup>(19)</sup> Briefly, LNCaP cells or frozen ventral prostate tissues were homogenized in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail [Complete, Roche, Mannheim, Germany]) and subjected to immunoblot analysis using standard techniques. The antibodies used were Cyclin D1 and androgen receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cleaved caspase 3, cleaved caspase 7, Erk1/2, phospho-Erk1/2, p38 MAPK and phospho-p38 MAPK (Cell Signaling Technology, Boston, MA, USA), prostate-specific antigen (DAKO, Tokyo, Japan) and  $\beta$ -actin (Sigma-Aldrich, St. Louis, MO, USA). The density of the bands was semi-quantified using ImageJ (version 1.42q, National Institute of Health, Bethesda, MD, USA).

**Immunohistochemistry.** Deparaffinized sections were incubated with antibodies for Ki-67 (Novocastra Laboratories, Newcastle, UK) and SV40 T antigen (Santa Cruz Biotechnologies). Apoptotic cells in the prostate were detected using an *In Situ* Apoptosis Detection kit (TUNEL method) according to the manufacturer's instructions (Takara Bio, Ohtsu, Japan). Labeling indices were determined as the positive percentage for Ki-67 or TUNEL by randomly picking eight fields of view in the whole ventral/lateral prostate and counting over 1000 prostate epithelial cells under a microscope at high magnification.

**Cell proliferation assay.** Cell proliferation of LNCaP cells was assessed by manual counting under trypan blue staining. Briefly, LNCaP cells were seeded in 96-well plates at 10 000

cells/well in 200  $\mu\text{L}$  of RPMI media; PCC, C3G, Pg3G or P3G were added 24 h after seeding and the cells were incubated for 72 h; and the cells were removed from the plate with trypsin-EDTA and counted.

**RNA extraction, cDNA preparation, and quantitative real-time PCR.** Total RNAs of LNCaP cells or frozen prostate tissue was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and reverse-transcribed with the ThermoScript first-strand synthesis system (Invitrogen Corporation, Carlsbad, CA, USA). Real-time RT-PCR was performed using Syber Premix Ex Taq II (Takara) in a LightCycler (Roche Diagnostics GmbH). The primers used were: human GAPDH,  $60^\circ\text{C}$ , 5'-AA CGGATTTGGTCGTATTGG-3' and 5'-CATACTTCTCATGG TT-CACA-3'; human Cyclin D1,  $60^\circ\text{C}$ , 5'-CCGAGAAGCT GTGCATCTAC-3' and 5'-CAGGTTTCAGGCCTTGCCTG-3'; rat GAPDH,  $59^\circ\text{C}$ , 5'-GAATGGGAAGCTGGT-CATCA-3' and 5'-TGGATGCAGGGATGATGTTC-3'; rat probrasin,  $60^\circ\text{C}$ , 5'-ACTTCCGTCGATTGAGTGT-3' and 5'-GTAAACGTCTT GGGATCTCC-3'; rat GK11,  $59^\circ\text{C}$ , 5'-GCAGCACCAAACCC CTGGAT-3' and 5'-TGAGATCTGTACCTTCTCA-3'.

**Cell cycle analysis.** LNCaP cells were seeded in 6-well plates at 150 000 cells/well. Purple corn color, C3G or Pg3G were added 24 h after seeding, incubated for 72 h, collected and analyzed with propidium iodide (Guava cell cycle reagent; Guava Technologies, Hayward, CA, USA) according to the Guava Cell Cycle Assay protocol. The cell cycle phase distribution was determined on a Guava PCA Instrument using CytoSoft Software.

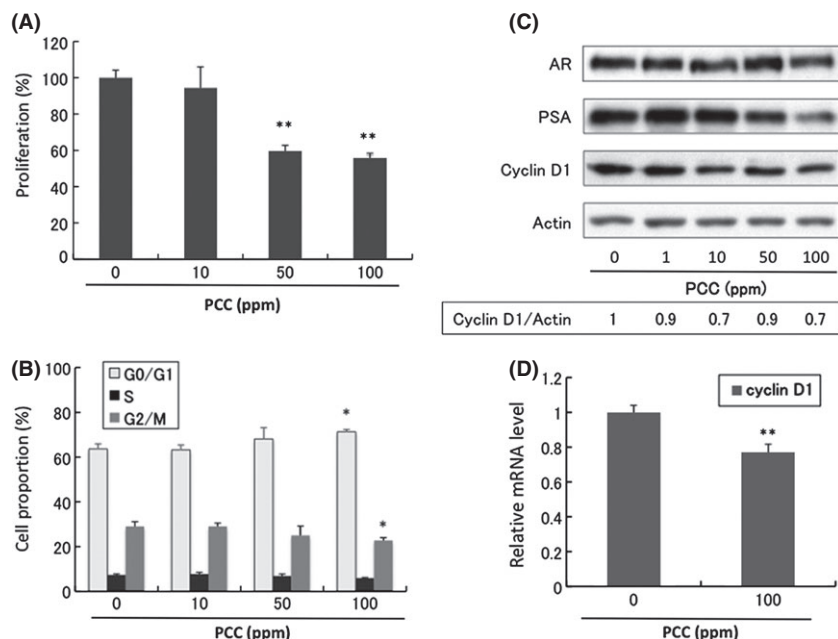
**Reporter gene assays.** LNCaP cells were transfected with the pGL3/PSA-Luc using Nucleofector II. 24 h later, 5 nM DHT and/or PCC were added. After 48 h incubation, the cells were lysed with the buffer supplied in the kit. Luciferase assays were conducted using the dual-luciferase reporter assay system (Promega, Madison, WI, USA), and the pHRL-TK vector (Promega) as an internal control, according to the manufacturer's protocol. Data shown are means and SD of four independent data points.

**Statistical analysis.** All data presented are mean  $\pm$  SD values. Statistical comparisons were performed with one-way ANOVA followed by Dunnett's test. Correlations were assessed by Spearman correlation coefficient analysis. A  $P$ -value of  $< 0.05$  was considered to be significant. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

## Results

**PCC inhibition of LNCaP proliferation and slowing of the cell cycle.** When the androgen-dependent cell line LNCaP was incubated with increasing levels of PCC for 72 h, the proliferation of the cells was inhibited in a dose-dependent manner (Fig. 1A). Cell cycle analysis showed that PCC increased the proportion of cells in G0/G1 slightly but significantly (Fig. 1B). Western blotting showed a 15–30% decrease in the protein level of Cyclin D1 in the PCC treated cells (Fig. 1C), and reverse-transcription PCR also showed that the mRNA level of Cyclin D1 was significantly decreased by PCC (Fig. 1D). Purple corn color did not affect androgen receptor (AR) expression, but PSA expression was dramatically decreased (Fig. 1C). Since the PSA gene is a target of AR, we used a luciferase assay to examine the influence of PCC on the activity of the PSA promoter. Purple corn color inhibited functional AR transcriptional activity in a dose-dependent manner (Fig. S2).

**No toxic effects of PCC were observed in the TRAP rat model.** Body weights, relative organ weights (ventral prostate, liver and kidney), and food consumption were not affected by administration of PCC in the diet to TRAP rats (Table S1). The average PCC intakes were 25 mg/rat per day and 267 mg



**Fig. 1.** Effects of purple corn color (PCC) on LNCaP cells. (A) Inhibitory effects on LNCaP cell proliferation (72 h) ( $n = 3$ ). (B) Effects on the cell cycle of LNCaP cells ( $n = 3$ ). (C) Protein changes assessed by Western blotting analysis of LNCaP cells after incubation with PCC for 72 h. The density of the bands of Cyclin D1 and actin were semi-quantified by ImageJ. (D) mRNA levels of Cyclin D1 analyzed by reverse-transcription polymerase chain reaction ( $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

/rat per day for the 0.1% and 1% PCC groups (Table S1). Purple corn color did not have any effect on the serum levels of testosterone or estradiol (Table S1).

**PCC inhibition of prostate carcinogenesis in the TRAP rat model.** With the expression of SV40T antigen under the control of AR, TRAP rats developed prostate cancer with high incidence. In the end of the experiment, all the acini in both ventral and lateral prostate of TRAP rats had developed pre-cancerous or cancerous lesions. Lesions were divided into three stages: LG-PIN, HG-PIN, and adenocarcinoma (Fig. S3). The total percentage of LG-PIN, HG-PIN and adenocarcinoma is 100%. In this study, adenocarcinomas were observed in ventral prostate of all the rats, that is, the incidence of adenocarcinoma was 100% in all three groups. However, the rats consuming PCC had a lower percentage of adenocarcinoma and a higher percentage of LG-PIN (Table 1), which suggests that PCC retarded the progression of PCa and more acini lesions remained in the relatively benign LG-PIN stage. The increased LG-PIN percentage and decreased adenocarcinoma percentage showed a strong correlation to the dose of PCC. In the lateral prostate, we also observed this retardation of PCa progression. Of critical importance in this study is that PCC also significantly decreased the incidence of adenocarcinoma in the lateral prostate (Table 1). These results suggest that PCC inhibited tumorigenesis in the prostates of TRAP rats.

**PCC inhibition of the cell growth pathways in the TRAP rat model.** Carcinogenesis in the TRAP rat is induced by SV40T antigen expressed under the control of the probasin promoter, which is regulated by the AR. Since PCC downregulated AR activity *in vitro*, we examined whether SV40T expression was downregulated by PCC. Immunohistochemical analysis showed that there was no overt inhibition of SV40T expression by PCC (Fig. 2A,B). We used RT-PCR to examine mRNA levels in the VP of the androgen responsive genes probasin and GK11, an ortholog of human PSA. These results also indicated that AR activity was not inhibited by PCC (Fig. 2C).

Figure 3A shows that the Ki67 index was decreased by PCC in both VP and LP. On the other hand, the TUNEL staining index was not affected by PCC (Fig. 3B), in agreement with our *in vitro* studies in which no apoptosis was observed (data not shown).

Immunoblotting demonstrated factors involved in cell growth pathways, Erk1/2 and p38 MAPK phosphorylation and Cyclin D1, to be downregulated by PCC. In agreement with the results of TUNEL staining, PCC had no effect on the levels of cleaved caspases 3 or 7 (Fig. 3C).

**Search for active compounds in PCC.** The compounds that give PCC its purple color are anthocyanins. C3G, Pg3G and P3G are the three major components of PCC. When their effects on LNCaP cells were tested, C3G and Pg3G dose-dependently inhibited the proliferation of LNCaP cells, while P3G had no effect (Fig. 4A). The differences of the chemical structures of these three chemicals (Fig. S1) suggest that the hydroxyl radical may play an important role in the inhibitory activity on PCa. Both C3G and Pg3G upregulated AR expression. However, PSA expression, an indicator of AR activity, remained the same (Fig. 4B). This effect on AR activity is in contrast to the effect of PCC *in vitro* but similar to that *in vivo*. Both C3G and Pg3G decreased the expression of Cyclin D1 (Fig. 4C), while increasing the proportion of cells in G0/G1 (Fig. 4D,E), again in line with the PCC effect.

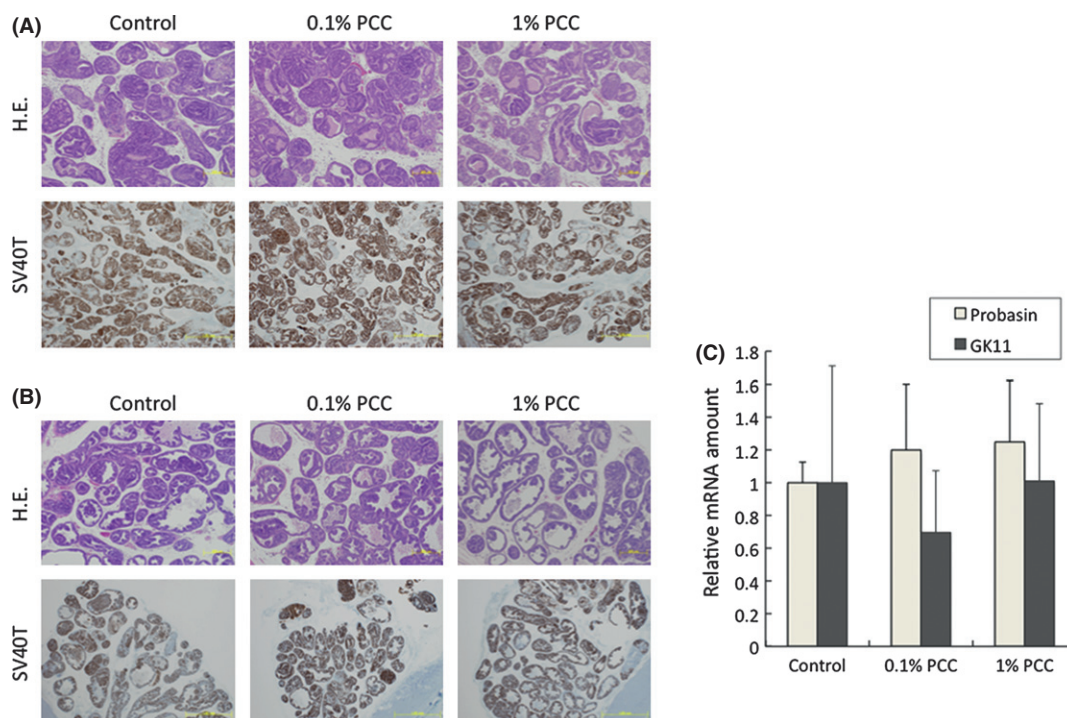
## Discussion

Purple corn color is reported to have anti-cancer effect on colon and breast cancer,<sup>(9,10)</sup> and it also has attenuating effects on some life style diseases, for example, hypertension, hyperglycemia, and diabetes.<sup>(11,12)</sup> Like breast cancer, PCa is hormone-related. Prostate cancer is also closely associated with a high-fat diet.<sup>(20,21)</sup> Finally, in the TRAP model, hypertension is positively associated with PCa development.<sup>(22)</sup> Therefore, we investigated the possibility that PCC could have inhibitory effects on PCa. The results of our experiments, both *in vitro* and *in vivo*, supported this hypothesis. Purple corn color not only showed antiproliferative effects on an androgen-dependent PCa cell line, it also inhibited prostate carcinogenesis *in vivo*. Importantly, dietary PCC did not have any observable toxic effects: there were no significant changes in the final body weights or relative liver or kidney weights in rats fed PCC in their diets. This suggests that PCC could be used as a long-term dietary supplement for chemoprevention of PCa. The success of PCC in the TRAP

**Table 1. Quantitative evaluation of neoplastic lesions in prostates of TRAP rats treated with PCC**

	No. of animal	Incidence of adenocarcinoma	Proportion of acini in different stages of carcinogenesis (%)		
			LG-PIN	HG-PIN	Adenocarcinoma
<b>Ventral prostate</b>					
Control	13	13 (100%)	4.4 ± 2.8	89.2 ± 3.5	6.4 ± 4.0
0.1% PCC	12	12 (100%)	5.9 ± 3.4**	90.6 ± 3.8	3.5 ± 1.7***
1% PCC	11	11 (100%)	9.1 ± 3.9**	88.2 ± 3.4	2.7 ± 2.0***
<b>Lateral prostate</b>					
Control	13	12 (92%)	24.7 ± 12.4	72.8 ± 11.7	2.5 ± 2.8
0.1% PCC	12	7 (58%)	17.8 ± 5.6	81.2 ± 5.6	1.0 ± 1.0**
1% PCC	11	3 (27%)*	21.1 ± 6.8	78.0 ± 6.2	0.9 ± 1.6**

AC, adenocarcinoma; HG, high grade; LG-PIN, low grade prostatic intraepithelial neoplasia. \**P* < 0.01 versus control (Dunnett's test). \*\**P* < 0.01 and \*\*\**P* < 0.001 versus control, respectively (Spearman's rank correlation coefficient analysis).



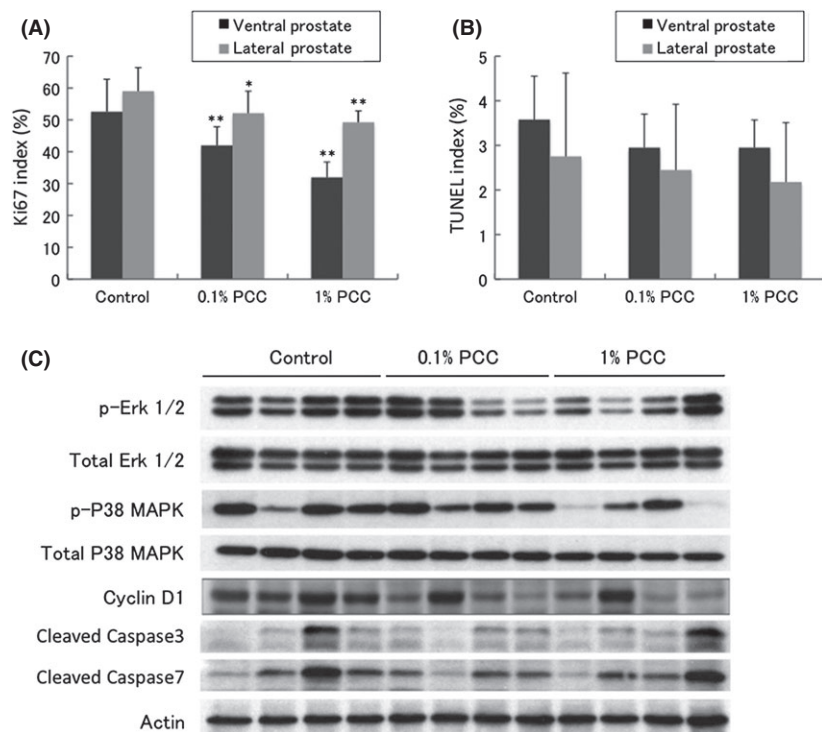
**Fig. 2.** Purple corn color (PCC)-mediated inhibition of carcinogenesis is not due to down regulation of androgen receptor (AR) activity. (A) H&E staining (4 × magnification) and SV40T antigen expression (4 × magnification) in the ventral prostate. (B) H&E staining (4 × magnification) and SV40T antigen expression (4 × magnification) in the lateral prostate. (C) mRNA of the ventral prostate was used for reverse-transcription polymerase chain reaction. mRNA levels of probasin and GK11 (the rat ortholog of human PSA), which are AR target genes, were checked. Scale bars, 500 μm.

rat model demonstrates that PCC may inhibit prostate carcinogenesis not only in a simple *in vitro* tissue culture system but also in the complex system of living experimental animals.

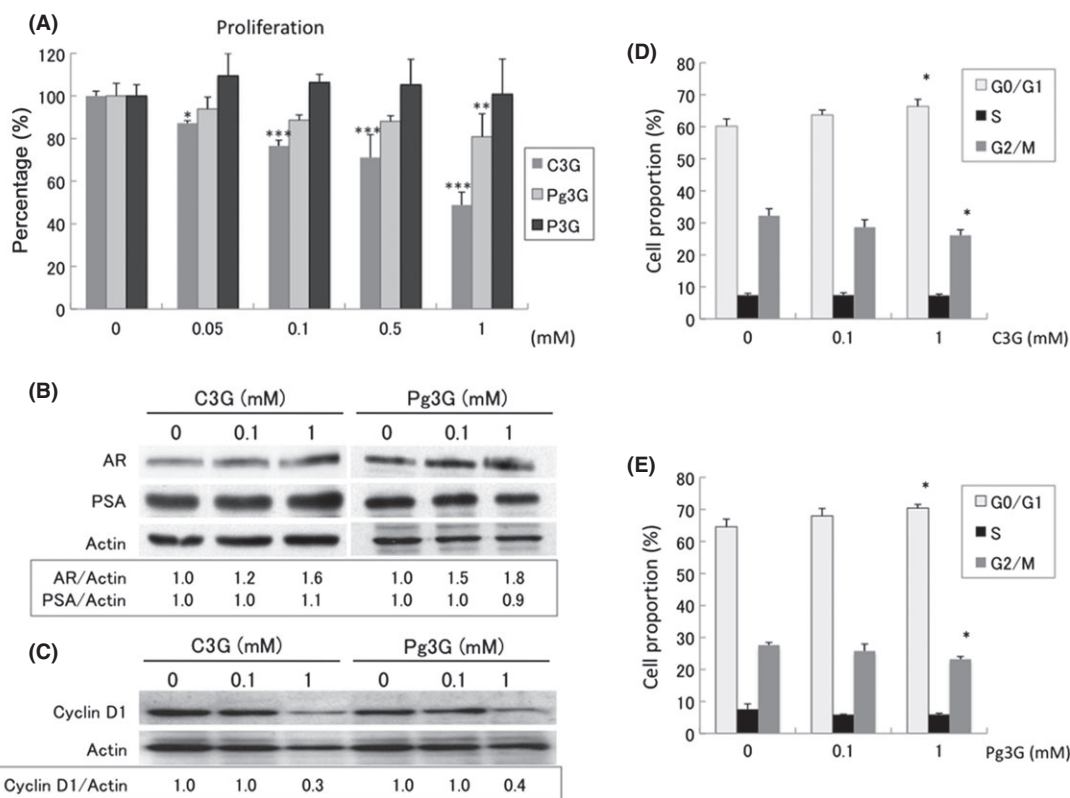
To date, an enormous effort has been made to find means to prevent PCa, but an ideal chemopreventor active by itself has yet to be found. The trend has therefore been to put chemicals that target different pathways together to form a “cocktail” that would be more effective. Our laboratory has been focused on looking for PCa chemopreventors. We previously reported that resveratrol could inhibit PCa genesis by targeting the AR pathway,<sup>(19)</sup> while  $\gamma$ -tocopherol exerts inhibitory effects through activation of caspase-signaling.<sup>(23)</sup> In the present study, we found that PCC targets cell growth. A reasonable expectation is that combining PCC, resveratrol and  $\gamma$ -tocopherol could have a greater effect than

using any of the compounds singly. This is a direction for future studies.

To better understand the chemopreventive effects of PCC on PCa, identifying the active components is essential. Cell proliferation assays here indicated that both C3G and Pg3G dose-dependently inhibited the growth of LNCaP. Similarly to PCC, both C3G and Pg3G decreased the expression of Cyclin D1 and increased the percentage of LNCaP cells in G0/G1. The increased percentage of cells in G0/G1 was about 1.1 times compared to the control. Importantly, the cell proliferation was inhibited by approximately 50%. This suggests that while the effect of C3G and Pg3G on the cell proliferation is rather small, the cumulative effect over time can be substantial. Intriguingly, although the PCC mixture downregulated AR activity, C3G and Pg3G did not show the same effect. Notably, PCC inhibited carcinogenesis in the TRAP model without



**Fig. 3.** Effects of purple corn color (PCC) on Ki67 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) indices in the prostate. (A) Purple corn color decreased the Ki67 index significantly in both the ventral and lateral prostate (samples from all the rats were used for analysis). (B) Purple corn color did not affect the TUNEL index in either the ventral or lateral prostate (samples from all the rats were used for analysis). (C) Western blot of proteins related to cell growth and apoptosis using samples from the ventral prostate.



**Fig. 4.** Anthocyanins in the purple corn color (PCC) mixture, cyanidin-3-glucoside (C3G), pelargonidin-3-glucoside (Pg3G) and peonidin-3-glucoside (P3G), were tested using LNCaP cells. (A) Effects of C3G, Pg3G and P3G on LNCaP cell proliferation. (B,C) Findings of Western blotting. The density of the bands of androgen receptor (AR), PSA and actin were semi-quantified by ImageJ. (D) Effects of C3G on the cell cycle of LNCaP cells. (E) Effects of Pg3G on the cell cycle of LNCaP cells. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

downregulating AR activity. Therefore, it is likely that PCC does not inhibit PCa through downregulation of AR. The downregulation observed with the PCC mixture *in vitro* might

be a byproduct of other compounds. Taken together, these results suggest that C3G and Pg3G are the probable active compounds contained in PCC.

Carcinogenesis is a complex and long-term process. Therefore, finding a chemopreventor and using it to inhibit the progression of PCa will be of general benefit. Our study has proved: (i) the safety of relatively long-term PCC consumption; (ii) PCC can inhibit PCa both *in vitro* and *in vivo*, and modulation of cell growth pathways may possibly be involved in suppressive effects of PCC. Other studies have reported that C3G and Pg3G, the active compounds in PCC, have antioxidant and free-radical-scavenging effects, which may protect cells from oxidative damage and reduce the risk of diabetes, cardiovascular diseases and cancer.<sup>(24–27)</sup> With further study, we can expect to determine whether the mechanism of PCC inhibition of PCa also involves these pathways. Modulation of multiple pathways can increase the chance of PCC effectively preventing PCa in humans. Taking the available information

into account, PCC, a widely used food colorant, appears to be a promising chemopreventor for PCa.

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## Disclosure Statement

The authors have no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** The chemical structures of C3G, Pg3G and P3G.

**Fig. S2.** PCC down regulated the activity of the PSA promoter.

**Fig. S3.** The different stages of carcinogenesis in the prostate of TRAP rat.

**Table S1.** Final body and organ weights, serum hormone levels, and average PCC intake.