Purple corn color suppresses Ras protein level and inhibits 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in the rat

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Anthocyanins belong to the class of phenolic compounds collectively named flavonoids. Many anthocyanins are reported to have inhibitory effects on carcinogenesis. Purple corn color (PCC), an anthocyanin containing extract of purple corn seeds, is used as a food colorant. The major anthocyanin in PCC is cyanidin 3-*O*-β-D-glucoside (C3-G). The present study was conducted to assess the influence of dietary PCC on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in rats. PCC significantly inhibited DMBA-induced mammary carcinogenesis in human c-Ha-*ras* proto-oncogene transgenic (Hras128) rats and in their non-transgenic counterparts. PCC and C3-G also inhibited cell viability and induced apoptosis in mammary tumor cells derived from Hras128 rat mammary carcinomas. At the molecular level, PCC and C3-G treatment resulted in a preferential activation of caspase-3 and reduction of Ras protein levels in tumor cells. It is proposed that C3-G could act as a chemopreventive and possibly chemotherapeutic agent for cancers with mutations in *ras*. Secondly, the *in vitro–in vivo* system used in this study can be utilized for screening for cancer preventive compounds that act via Ras down-regulation. (*Cancer Sci* 2008; 99: 1841–1846)

Anthocyanins are found throughout the plant kingdom and impart purple, blue and red color to fruits and vegetables. They belong to the class of phenolic compounds collectively named flavonoids. Anthocyanins are naturally present as glycosides having glucose, galactose, rhamnose, xylose or arabinose attached to the aglycon nucleus; the sugar-free aglycon nucleus is known as anthocyanidin. Several hundred anthocyanin species exist depending on the glycoside structure. It is generally accepted that anthocyanin food colors do not exert obvious toxicity, teratogenicity or mutagenicity and, indeed, anthocyanins may inhibit mutagenesis in the Ames test.[1–3] In studies testing the effects of anthocyanins on carcinogenesis, inhibitory effects of anthocyanins have been reported.[4–9]

PCC (Maize morado color) is extracted from the seeds of purple corn, *Zea mays* L., and is used as a beverage colorant (as Chica Morada) in Latin America, especially in Peru. PCC has been shown to inhibit azoxymethane-induced colon tumors in rats.[4] PCC contains six anthocyanins; the major anthocyanin found in PCC is cyanidin 3-*O*-β-D-glucoside (C3-G). *In vitro*, C3-G reacts with peroxyradicals and is converted into the oxidation products 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran and protocatechuic acid (PC); PC is also a radical scavanger.[10] Thus, after C3-G reacts with biological radicals, a second radical scavenger is produced. C3-G also gives rise to PC *in vivo*: after oral administration of C3-G, both C3-G itself and PC are found in the plasma.[11] The plasma C3-G concentration reaches a maximum at 30 min after single oral administration. The half life of plasma C3-G is about 2 h.[11] Therefore, C3-G in the diet is expected to improve the body's antioxidant capability and inhibit carcinogenesis *in vivo*.

We have established a rat line carrying copies of the human c-Ha-*ras* proto-oncogene under the regulation of its own promoter region (Hras128). This line is highly susceptible to *N*-methyl-*N*-nitrosourea (MNU)- and DMBA-induced mammary carcinogenesis.[12–14] Tumors develop in almost all females within as short a period as 8–12 weeks after a single MNU or DMBA treatment. The animals have also been found to be susceptible to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder,[15] DMBA-induced skin,[16] and 4-nitroquinoline 1-oxide-induced tongue[17] carcinogenesis. This model can be used for the short-term assay of test compounds including chemopreventive compounds,[18] genotoxic compounds[19] and non-genotoxic promoting agents.[20] In the present study, this short-term tumor model was employed to investigate the effects of PCC on DMBA mammary carcinogenesis. Non-transgenic rats were then used to confirm the effects that PCC had on transgenic rats. PCC inhibited mammary carcinogenesis in both transgenic and non-transgenic rats.

Recently, we established cell lines from mammary carcinomas induced by DMBA in Hras128 rats.[21] These cells can be utilized for mechanistic analysis of compounds showing a modifying influence on mammary carcinogenesis in Hras128 rats. Accordingly, we used the cell lines for the analysis of the mechanism by which PCC inhibited carcinogenesis.

Materials and Methods

Animals. Female c-Ha-*ras* transgenic (Hras128, Tg) and non-transgenic (non-Tg) rats were bred by CLEA Japan, Tokyo, Japan. They were maintained in plastic cages in an air-conditioned room with a 12-h light/12-h dark cycle. In total, 62 Tg and non-Tg rats received a single dose of DMBA (Tokyo Chemical Industry, Tokyo, Japan) (25 mg/kg body weight) by gavage at 7 weeks of age. One day thereafter, they were placed on powdered basal diet MF (Oriental Yeast, Tokyo, Japan) containing either purple corn color (PCC) (San-Ei Gen F.F.I., Osaka, Japan) or no supplement. PCC was prepared as described previously.[4] The specifications of PCC used in this study were as follows: trade name San RED No.5, lot No. 040421, purity 33.7% as anthocyanin concentration. Gross observation and palpation of the mammary gland were regularly performed to monitor the development of mammary tumors after DMBA

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Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; PCC, purple corn color; C3-G, cyanidin 3-*O*-β-D-glucoside; PC, protocatechuic acid.

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treatment. The surviving animals were killed by exsanguination under deep ether anesthesia at the end of week 8 for Tg and week 22 for non-Tg rats. The numbers of visible tumors were recorded before they were measured and sampled for histological examination. Values are expressed as average tumor weight of the total tumors for each rat. Body and liver and kidney weights were also recorded. The experiments were conducted according to the 'Guidelines for Animal Experiments of the Nagoya City University Graduate School of Medical Sciences'.

Cell culture. Rat mammary carcinoma cells (C3, C11 and C17),[21] which were established from DMBA-induced tumors in Hras128 rats, were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Cells were seeded in 96-well plates, and then incubated with PCC, C3-G (Extrasynthese, Genay Cedex, France) or PC (Wako Pure Chemicals, Osaka, Japan) for 1 day. Cell viability was measured using a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

In some experiments, the cells were grown to confluency, then rendered quiescent by incubation in DMEM containing 0.5% FCS. After serum starvation for 48 h, the cells were treated with 10% FCS to initiate signaling cascades.

Western blot. Cells or tissues were lyzed in a minimal volume of lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 1 mM NaF and protease inhibitors [protease inhibitor cocktail, Sigma, Saint Louis, MO, USA]). Total proteins were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). The blots were incubated with primary antibodies after blocking with 3% non-fat milk. The blots were then washed and incubated with horseradish peroxidase-conjugated antirabbit and antimouse immunoglobulin G (IgG) antibodies (Southern Biotechnology Associates, Birmingham, AL, USA). The bound antibodies were detected using enhanced chemiluminescence (ECL) plus Western blotting detection system (GE Healthcare Bio-sciences, Piscataway, NJ, USA). Detection of activated Ras protein was performed using a Ras activation assay kit (Upstate, Lake Placid, NY, USA) as described previously.[22,23]

The following antibodies were used: pan-Ras (1/4000, clone RAS10; Upstate), extracellularsignal- regulated kinase (Erk) 1/2 (1/50 000, 06–182; Upstate), phospho-ERK 1/2 (1/1000, #9106; Cell Signaling Technology, Danvers, MA, USA), Akt (1/1000, #9272; Cell Signaling Technology), phospho-Akt (Ser473) (1/1000, #4051; Cell Signaling Technology), caspase-3 (1/1000, #9662; Cell Signaling Technology), cleaved caspase-3 (1/1000, #9661; Cell Signaling Technology), caspase-8 (1/1000, H-134; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (1/10 000, A5441; Sigma, Saint Louis, MO, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of apoptotic cells. Apoptotic cells were detected by TUNEL assay using an *In situ* Apoptosis Detection Kit (Takara Bio, Otsu, Japan) according to the manufacture's instructions. Fluorescent nuclear staining was performed with TO-PRO-3 iodide (Molecular Probes, Eugene, OR) at a dilution of 1/1000. A confocal microscope FLUOVIEW FV300 (Olympus, Tokyo) was utilized for imaging.

Real-time polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and the RNA was reverse-transcribed using Superscript III Reverse Transcriptase with Random primers (Invitrogen) according to the manufacturer's instructions. PCR amplification was carried out using SYBR *Premix Ex Taq* and the Smart Cycler II System (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. All PCR amplifications were done for 40 cycles and a melt curve analysis was used to examine the specificity of the amplified products. The following primers were used: H*ras*, 5′-CAGTACAGGGAGCAGATCAA-3′ and 5′-AGCACACACTTGCAGCTCAT-3′; K*ras*, 5′-GCGTAGGCAAG AGTGCCTTGA-3′ and 5′-GACCTGCTGTGTCGAGAATATCCA-3′; N*ras*, 5′-AGCAGTGAGGATGGCACTCAAG-3′ and 5′-GATGTC AGAACCAGGGCATCAG-3′; β-*actin*, 5′-CCGTAAAGACCT CTATGCCAACA-3′ and 5′-CGGACTCATCGTACTCCTGCTT-3′.

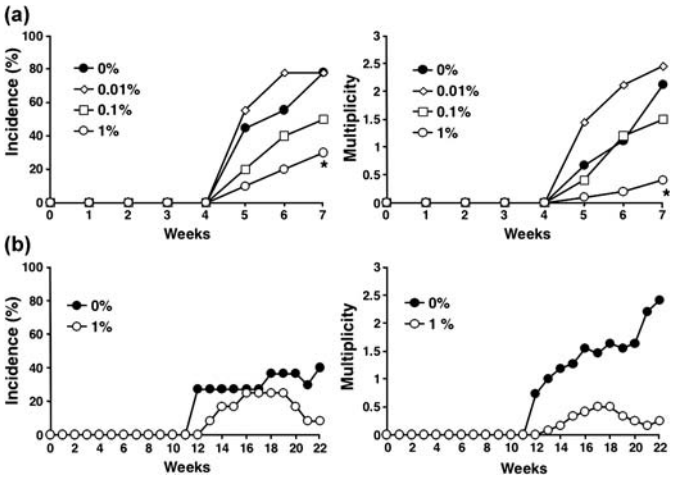

Fig. 1. Periodic observation of palpable mammary tumors after 7,12-dimethylbenz[*a*]anthracene (DMBA) treatment in female (a) transgenic (Tg) and (b) non-Tg rats fed purple corn color. The *x*-axis indicates weeks after DMBA treatment. $*P < 0.05$ as compared with the 0% group.

Statistics. All the average values are expressed as the means ± SD. Analysis was performed using the JMP software package (SAS Institute, Cary, NC, USA). Fisher's exact test was conducted for tumor incidence data. Dunnett's *t*-test was conducted for body and organ weight. Mann–Whitney tests were conducted for tumor weight data and multiplicity data.

Results

General observations in the animal experiments. Modifying effects of PCC on DMBA-induced mammary carcinogenesis in female Tg and non-Tg rats were examined. Two rats died in the non-Tg 0% group before the experiment was terminated. There were no consistent significant differences noted in the intake of food or the weights of body, liver or kidneys (data not shown). Coloring of black feces, considered to be related to PCC treatment, was noted in rats fed 1% PCC.

Effects of PCC on mammary tumor induction. In the Tg rats, palpable mammary tumors were first observed at 5 weeks after administration of DMBA. After 5 weeks, the incidence increased rapidly. Tumor incidence and multiplicity increased with time, but was suppressed in a dose-dependent manner in animals fed PCC (Fig. 1a). Final mammary tumor incidences and multiplicity data determined by histological examination are summarized in Table 1. Most of the mammary tumors were diagnosed as adenocarcinomas. The weight of the mammary tumors was significantly lower in the 1% group compared with the 0% group ($P < 0.05$). PCC significantly decreased the incidence of middle-sized (0.5–2.0 g) mammary tumors in Tg rats ($P < 0.05$). It was not statistically significant, but the number of large-sized (>2.0 g) mammary tumors was also decreased by PCC. On the other hand, the number and incidence of smaller-sized (<0.5 g) mammary tumors was not suppressed by PCC, indicating that PCC is not able to inhibit the emergence of mammary tumors in Tg rats.

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Table 1. Inhibitory effects of purple corn color on mammary tumor induction

	Dose (%)	No. of rats	Tumor incidence and multiplicity								Weight of tumor (g)
			~0.5 g†		0.5 g ~2 g†		2 g~†		Total		
			Incidence (%)	No./rat	Incidence (%)	No./rat	Incidence (%)	No./rat	Incidence (%)	No./rat	
Tg	0	9	8 (88.9)	3.4 ± 2.1	8 (88.9)	1.8 ± 1.7	4 (44.4)	1.0 ± 1.4	8 (88.9)	6.2 ± 4.2	1.01 ± 1.34
	0.01	9	9 (100)	5.3 ± 4.4	8 (88.9)	2.9 ± 2.4	6 (66.7)	1.2 ± 1.1	9 (100)	9.4 ± 5.5	0.99 ± 1.52
	0.1	10	6 (60.0)	3.1 ± 4.0	6 (60.0)	1.9 ± 3.1	4 (40.0)	0.8 ± 1.1	8 (80.0)	5.4 ± 5.4	1.06 ± 1.68
	1	10	10 (100)	3.9 ± 3.5	2 (20.0)**	0.6 ± 1.3	3 (30.0)	0.4 ± 0.7	10 (100)	4.9 ± 4.6	0.69 ± 1.64*
non-Tg	0	10	6 (60.0)	4.1 ± 8.6	4 (40.0)	2.6 ± 5.1	3 (30.0)	0.9 ± 1.9	7 (70.0)	7.6 ± 15.3	0.90 ± 1.17
	1	12	2 (16.7)	1.0 ± 2.7	1 (8.3)	0.16 ± 0.58	0 (0)	0	2 (16.7)*	1.2 ± 3.2*	0.24 ± 0.31***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, as compared with 0% group; †tumor weight. Tg, transgenic; non-Tg, non-transgenic.

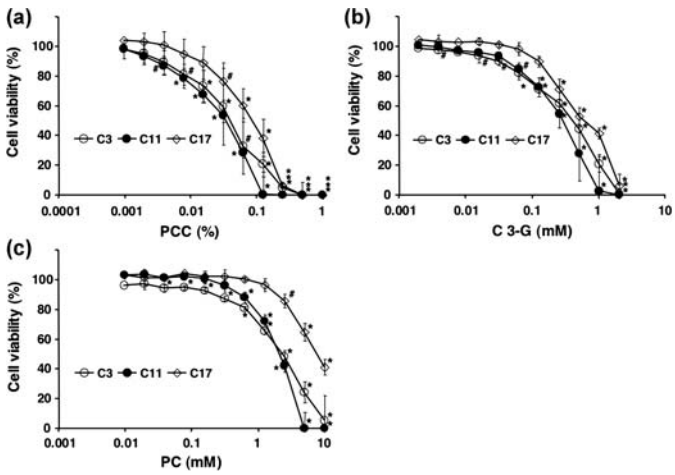

Fig. 2. Cytotoxicity induced by purple corn color (PCC) in mammary tumor cells. Rat mammary tumor cells (C3, C11 and C17) were established from 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced tumors in a transgenic (Tg) rat. These three cell lines have mutated human Ha-*ras* but not rat Ha-*ras*. Cells were incubated with each compound for 2 days. Dose dependent inhibition of cell proliferation by (a) PCC, (b) cyanidin 3-*O*-β-D-glucoside (C3-G) and (c) protocatechuic acid (PC). #$P < 0.05$, * $P < 0.01$ as compared with no treatment cells.

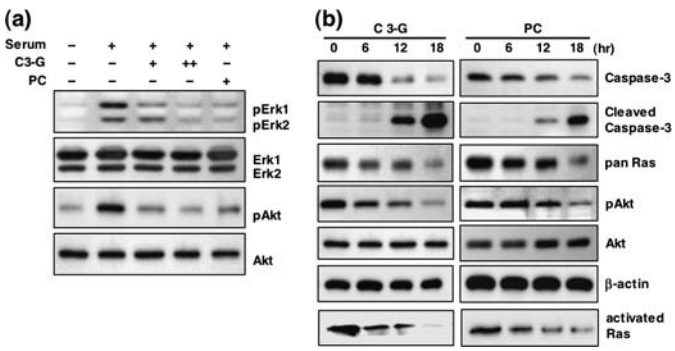

Fig. 3. Cyanidin 3-*O*-β-D-glucoside (C3-G) and protocatechuic acid (PC) inhibit Ras signaling and induce caspase-3 activation. (a) Effect of C3-G and PC on the activation of Erk1/2 and Akt in mammary tumor cells. C11 cells were serum starved for 48 h (0.5% FCS). The cells were incubated in the absence or presence of C3-G (+; 0.25 mM, ++; 0.5 mM) or PC (5 mM) in 0.5% FCS for 24 h prior to serum stimulation. After incubation in media with 10% FCS for 30 min, proteins were extracted. (b) C3-G and PC induced caspase-3 activation by degradation of Ras protein in mammary tumor cells. C11 cells were treated by C3-G (0.5 mM) or PC (5.0 mM) for the indicated time. The protein levels were assessed by Western blot. Activated Ras was precipitated by Raf-1 agarose and detected by antipan-Ras antibody.

Since PCC inhibited mammary carcinogenesis in Tg rats, we tested the inhibitory effect of 1% PCC on mammary carcinogenesis in non-Tg female rats. In non-Tg rats, palpable mammary tumors were first observed at week 12 and the incidence increased with time. PCC reduced mammary tumor incidence and multiplicity (Fig. 1b). Surprisingly, some palpable tumors regressed and became undetectable in rats fed 1% PCC. This suggests that PCC exhibits chemopreventive and chemotherapeutic activity. In non-Tg rats, the incidence and weight of total mammary tumors (Table 1) was significantly lower in the 1% group compared with the 0% group ($P < 0.05$ and $P < 0.005$ respectively). Importantly, not only was the overall number of mammary tumors decreased by PCC in non-Tg rats, but there was complete suppression of the formation of large tumors (2.0 g <) in the 1% PCC group. Most of the mammary tumors in the control group and all of the mammary tumors in the 1% PCC group were diagnosed as adenocarcinomas in non-Tg rats. There was no histological difference between the 0% and the 1% PCC group.

C3-G, and PC inhibit Ras signaling and reduce cell viability in mammary cancer cells. We examined the effects of PCC on the viability of rat mammary cancer cells. Data for the effects of PCC, C3-G, and PC on the viability of mammary cancer cell lines C3, C11, and C17 are summarized in Fig. 2. PCC, C3-G and PC decreased the viability of the cells dose dependently. PCC, C3-G and PC caused a 50% reduction in cell viability at concentrations of 0.051%, 0.395 mM, and 4.37 mM respectively. On the basis of these results, a concentration of 0.5 mM for C3-G and 5 mM for PC were selected for further analysis.

To examine possible effects of C3-G and PC on the Ras signaling pathway, Ras protein levels and phosphorylation of Erk and Akt were examined. Total Ras and activated Ras protein levels were decreased by treatment with C3-G or PC (Fig. 3b). Serum stimulation of serum-starved C11 cells led to activation of Erk and Akt after 30 min. When the cells were pretreated with C3-G or PC for 1 day before serum stimulation, activation of Erk and Akt by growth stimuli was suppressed (Fig. 3a). Although C3-G and PC reduce Ras protein levels and suppress Ras signaling, the gene expression level of *ras* was not changed by treatment with C3-G or PC (data not shown). This suggests that C3-G and PC reduce Ras protein levels by a post-transcriptional mechanism.

To determine whether C3-G and PC induce apoptosis, C11 cells were treated with C3-G or PC for 6, 12 and 18 h, and apoptotic cells were detected using TUNEL. TUNEL staining showed that C3-G and PC induced apoptosis in these mammary tumor cells (Fig. 4).

Caspase-3 is a key protease associated with DNA fragmentation and apoptosis. C3-G and PC induced activation of caspase-3 after

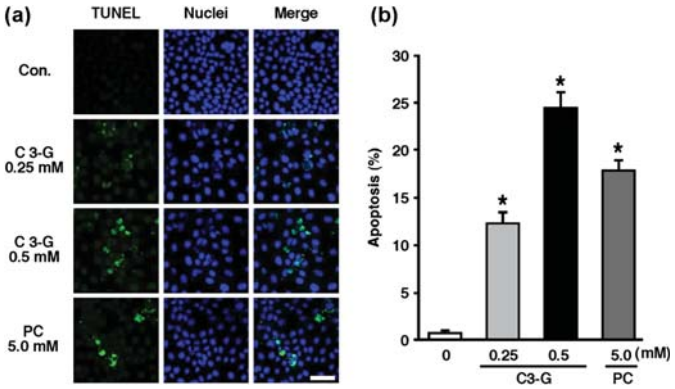

Fig. 4. Cyanidin 3-*O*-β-D-glucoside (C3-G) or protocatechuic acid (PC) induced apoptosis in mammary tumor cells. (a) Terminal deoxynucleotidyl transferase dUTP nick end (TUNEL) staining of mammary tumor cells treated with C3-G or PC. Bar = 50 μm. (b) The percentage of apoptotic cells was determined by counting TUNEL-positive cells from at least 3 fields. **P* < 0.01 as compared with control.

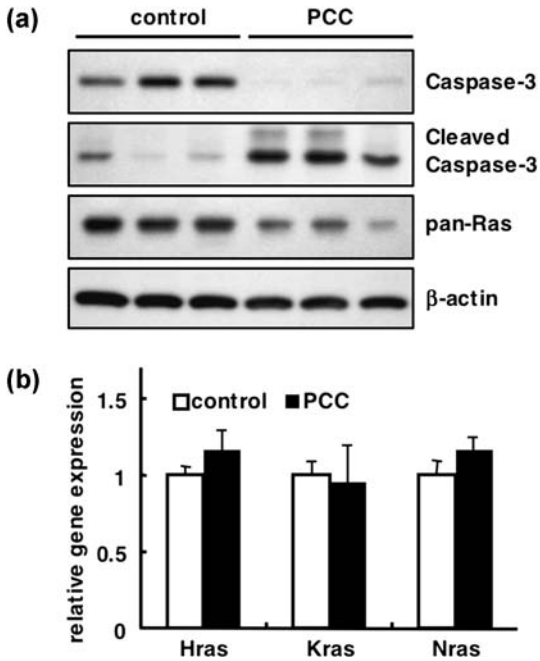

Fig. 5. Purple corn color (PCC) induced degradation of Ras protein in mammary tumors *in vivo*. Non-transgenic (Tg) rats were fed 0 or 1% PCC for 22 week after receiving 7,12-dimethylbenz[*a*]anthracene (DMBA). The tumors were removed, and proteins and RNA were extracted. (a) Western blot analysis: PCC reduced Ras protein level and activated caspase-3 in mammary tumors *in vivo*. (b) Quantitative real-time PCR analysis: The expression level of *ras* genes, H*ras* (the endogenous gene and the transgene), K*ras* and N*ras*, was not significantly changed in mammary tumors by PCC.

12 h (Fig. 3b). The level of cleaved caspase-3 correlated well with suppression of Ras protein expression and suppression of phosphorylation of Akt. C3-G and PC did not induce activation of caspase 8 (data not shown). This suggests that the TNFα/Fas signaling pathway is dispensable for C3-G and PC apoptotic activity.

PCC inhibits Ras and activates caspase-3 *in vivo*. In determining the true biological significance of a novel pathway, it is always important to confirm *in vitro* results in an *in vivo* context. We evaluated the effect of PCC on the stability of the Ras protein and activation of caspase-3 *in vivo*. As shown in Fig. 5a, PCC suppressed the level of Ras protein and induced the activation of caspase-3 in non-Tg rats. In line with *in vitro* data, total H*ras* (the endogenous gene and the transgene), K*ras* and N*ras* mRNA level was not changed by PCC (Fig. 5b).

Discussion

In the present study, we showed that PCC inhibits mammary tumor development in both cancer prone transgenic rats and their non-transgenic counter parts, and that this inhibition was clearly in line with induction of apoptosis in mammary cancer cell lines derived from the transgenic rats. A previous study reported that 5.0% PCC in the diet did not show any evidence of adverse effects.[4,24] Cyanidin 3-*O*-β-D-glucoside (C3-G) is the major anthocyanin in PCC. C3-G treatment decreased the number of skin tumors induced by 12-0-tetradecanoylphorbol 13-acetate (TPA) in DMBA-initiated mouse skin.[25] C3-G also reduced the size of A549 tumor xenograft growth and significantly inhibited metastasis in nude mice.[25] C3-G is converted to protocatechuic acid (PC) *in vivo,*[11] and PC possesses chemopreventive activities on liver, colon, oral and urinary bladder carcinogenesis.[26–30] Consistent with these observations, PCC inhibited development of mammary tumors in Tg and non-Tg rats.

We also demonstrated that Ras protein levels were decreased by treatment with PCC, C3-G and PC. One mechanism by which this could occur is via reduction of reactive oxygen species (ROS) levels. High ROS levels in conjunction with activation of ERK1/2 stabilize Ha-Ras protein by inhibiting proteasome degradation,[31] and anthocyanins show strong free radical scavenging and antioxidant activities.[32–34]

Down-regulation of Ras levels protects primary cells from inappropriate growth factor signaling, which may result in DNA damage, oxidative stress, and ultimately in apoptosis. However, Ras can activate seemingly contradictory intracellular pathways and in certain conditions Ras has antiapoptotic effects.[35–38] ROS generation is common in cancer cells experiencing oncogenic stimulation by factors such as Ras and Myc,[39,40] and, as noted above, high ROS levels stabilize the Ha-Ras protein. Therefore, PCC, C3-G, and PC might induce apoptosis in cancerous cells by decreasing Ras via reducing ROS levels.

Interestingly, it has been shown that Ha-Ras proteins are modified by mono- and diubiquitination which targets them to endosomes,[41] however, there are no reports of Ras polyubiquitination, which would target Ras proteins for proteasomal degradation. Therefore, it is likely that the effect of PCC on Ras protein expression is mediated through the proteasomal degradation of a factor that affects the stability of Ras.

Ras is found mutated in 30% of all tumors. Pancreatic cancer is the tumor type with the highest incidence of Ras mutations (90%), followed by colon (40%), thyroid (50%), and lung adenocarcinomas (30%).[42] Ras signaling to the PI3 kinase–Akt pathway is an important contributor to tumor cell survival. Aberrant Ras activation can occur as the result of several different cellular abnormalities, not only from mutation of the *ras* gene itself, but, for example, overexpression of the epidermal growth factor receptor. Amplification and/or overexpression of proto-oncogenes such as H-, K-, and N-ras, and neu/erbB-2/HER-2 are frequent events in mammary malignancies of humans,[43,44] and mice.[45] The level of active Erk is clearly elevated in the terminal end buds in the mammary glands of Hras128 transgenic rats.[46] Altogether, it is believed that 80% of all tumors have aberrant Ras pathway activation.[47]

Previous studies have shown that anthocyanidins inhibit TPA-induced Erk, AP-1 activation, and cell transformation.[48] Another flavonoid, silymarin, inhibited both ligand-induced and constitutive activation of erythroblastosis B1/epidermal growth factor receptor(erbB1) [Correction added 1 August 2008: in the preceding sentence erB1 has been corrected to erbB1] and its downstream signaling events.[49] These studies suggest that flavonids including anthocyanin have inhibitory effects on the Ras signaling

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cascade. Previous studies showed that treatment with limonene, an inhibitor of Ras protein isoprenylation, inhibited mammary tumor development,[50,51] and limonene is effective in preventing Ras-initiated mammary carcinomas. Therefore, inhibitors of the Ras signaling cascade might be good candidates for cancer preventive/therapeutic agents.

In the present study, we showed that the activation of Erk was inhibited by C3-G and PC. Our finding suggests that PCC induces apoptosis in mammary tumors by decreasing Ras protein levels. Withdrawal of oncogenic Ras results in regression of tumors.[52] The initial stages of regression involved marked apoptosis in the tumor cells and the surrounding endothelial cells. Apoptosis has been reported to play an important role in elimination of seriously damaged cells and tumor cells by chemopreventive agents.[53] Therefore, apoptosis-inducing agents are expected to be ideal anticancer drugs. Consequently, PCC and it constituent anthocyanidin C3-G are promising candidate chemopreventive and chemotherapeutic agents for cancers that have abnormally high Ras activity.

In summary, dietary administration of PCC significantly suppressed the development of DMBA-induced rat mammary adenocarcinomas. Such cancer protective effects mediated by PCC most likely relate to the modulation of cell proliferation and apoptosis in the mammary neoplastic lesions by reducing Ras protein levels. Furthermore, our *in vivo–in vitro* system, which utilizes Hras128 transgenic rats, non-transgenic rats and mutant Ras-expressing mammary cancer cell lines, can be used for screening for chemopreventive agents that act via suppressing the Ras signaling pathway.

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