

Black Raspberry Inhibits Oral Tumors in Mice Treated with the Tobacco Smoke Constituent Dibenzo(def,p)chrysene Via Genetic and Epigenetic Alterations



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ABSTRACT

We previously reported that the environmental pollutant and tobacco smoke constituent dibenzo[def,p]chrysene (DBP) induced DNA damage, altered DNA methylation and induced oral squamous cell carcinoma (OSCC) in mice. In the present study, we showed that 5% dietary black raspberry (BRB) significantly reduced ($P < 0.05$) the levels of DBP-DNA adducts in the mouse oral cavity with comparable effect to those of its constituents. Thus, only BRB was selected to examine if aberrant DNA methylation induced by DBP can be altered by BRB. Using comparative genome-wide DNA methylation analysis, we identified 479 hypermethylated and 481 hypomethylated sites ($q < 0.01$, methylation difference $>25\%$) between the oral tissues of mice treated with DBP and fed control diet or diet containing BRB. Among the 30 differential methylated sites (DMS) induced by DBP, we found DMS mapped to *Fgf3*, *Qrich2*, *Rmdn2*, and *Cbarp* were hypermethylated by BRB whereas hypomethylated by DBP at either the

exact position or proximal sites; DMS mapped to *Vamp3*, *Ppp1rB1*, *Pkm*, and *Zfp316* were hypomethylated by BRB but hypermethylated by DBP at proximal sites. In addition to *Fgf3*, 2 DMS mapped to *Fgf4* and *Fgf13* were hypermethylated by BRB; these fibroblast growth factors are involved in regulation of the epithelial–mesenchymal transition (EMT) pathway as identified by IPA. Moreover, BRB significantly reduced ($P < 0.05$) the tumor incidence from 70% to 46.7%. Taken together, the inhibitory effects of BRB on DNA damage combined with its effects on epigenetic alterations may account for BRB inhibition of oral tumorigenesis induced by DBP.

Significance: We provided mechanistic insights that can account for the inhibition of oral tumors by BRB, which could serve as the framework for future chemopreventive trials for addicted smokers as well as non- or former smokers who are exposed to environmental carcinogens.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common human cancer worldwide; oral squamous cell carcinoma (OSCC) is the most histologic type of this disease (1–3). Up to 77% of oral cancer cases are

diagnosed at an advanced stage (4) and early diagnosis of this disease has not improved over time; the survival rate is stagnant at approximately 50% but in general, it varies with the stage of the disease (4). About one-third of patients treated with surgery, radiotherapy, and chemotherapy (5) will experience local or regional recurrence and/or distant metastasis (6).

Exposure to exogenous carcinogens [tobacco smoke, smokeless tobacco, excess alcohol, human papillomavirus (HPV)] can account for 90% of OSCC (1, 2). Avoidance of risk factors has only been partially successful in preventing this disease, largely because of the addictive power of tobacco smoking and alcohol consumption. Therefore, novel or improved approaches to prevention and/or early detection are urgently needed for the management and control of this disease.

Prevention remains a desirable approach since treating cancers, including OSCC, at late stages even with improved targeted therapies continues to be a major challenge. Our approach to prevention is based on understanding the molecular mechanisms that account for the induction of OSCC by carcinogens found in the human environment (7). Preclinical animal models that employ environmental carcinogens, reflect tumor heterogeneity, and accurately reflect the cellular and

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molecular changes in the multi-step process of oral carcinogenesis in humans could provide a realistic platform. Thus, we introduced a novel OSCC mouse model using the environmental pollutant and tobacco smoke constituent dibenzo[*def*, *p*]chrysene, also known as dibenzo[*a,l*]pyrene (DBP), and its fjord region diol epoxide (DB[*a,l*]PDE; refs. 8, 9).

We found that both DBP and DB[*a,l*]PDE induced DNA damage in mouse oral tissues (7). The induction of DNA damage by these carcinogens stimulated a follow-up investigation aimed at determining their effects on *in vivo* mutagenesis in the oral cavity of Big Blue C57BL/6 mice (8, 9). Both carcinogens were powerful mutagens and induced mutation profiles in the *lacI* reporter gene similar to those observed in p53 gene in human HNSCC; specifically, 50% of DBP and DB[*a,l*]PDE-induced mutations are G:C→T:A and G:C→A:T substitution and about 30% of the mutations at AT base pairs and these percentages are similar to those found in the *P53* gene in human HNSCC (7, 10). Furthermore, we also showed that upregulation of p53 and COX-2 proteins was observed (8, 9). DBP also resulted in a significant upregulation of several inflammatory-related genes in the mouse oral tissue (11). Moreover, we showed that hypomethylation of *Fgf3* is a potential biomarker for early detection of OSCC in mice treated with DBP (12). *Fgf3* is among a large fibroblast growth factor superfamily genes, which are involved in numerous biological activities, including cell survival and regulation of epithelial–mesenchymal transition (EMT) pathway (13–15). Frequent amplification of *Fgf3* gene is observed in HNSCC (16–18).

The development of effective, safe, and easy to administer chemopreventive agents is urgently needed and continues to be an active area in the arena of cancer prevention research. Several sources of phytochemicals have been proposed but one that has shown great promise in cancer prevention in both preclinical and clinical investigations is black raspberry (BRB; refs. 7, 19–24). BRB has been shown to inhibit 7,12-dimethylbenz[*a*]anthracene (DMBA) induced cancer in the hamster cheek pouch (23) and 4-nitroquinoline-N-oxide (4-NQO)-induced tongue cancer in the rat (24). However, both DMBA and 4-NQO have never been detected in the human environment and in contrast to the hamster model, the human oral cavity lacks a cheek pouch. In addition, DMBA is known to induce H-ras mutations which are found in less than 5% of oral cancers in the Western world (7). Taken together, our mouse model offers a realistic platform to mechanistically study cancer prevention in the oral cavity at the genetic and epigenetic levels. Thus, in this study, we selected BRB powder as a whole food approach and its related constituents to initially determine their effects, using a short-term mouse bioassay, on DBP-induced DNA damage in the oral cavity; the results strongly suggest the protective role of BRB on DBP-induced DNA damage was comparable to other constituents and thus for practical reasons, only BRB was used in follow-up studies as a whole food approach to examine for the first time its effects on epigenetic regulation (DNA methylation) and tumorigenesis-induced by DBP in the mouse oral cavity.

Materials and Methods

Chemicals

We prepared DBP according to our published method (8). Structural characterization of this carcinogen was based on NMR and high-resolution mass spectral data, and its purity ($\geq 99\%$) was determined by HPLC. Protocatechuic acid (PA) and ferulic acid (FA) were purchased from Sigma-Aldrich Chemical Co. BRB powder was provided by Berri Products LLC. BRBE was provided by Dr. Stoner and prepared using a published method (25).

Animals

Species, strain, and sex (female B6C3F1 mice 6–8 weeks of age; The Jackson Laboratory) of the animals used in this study were selected on the basis of our previous report (8). Following 1 week of quarantine, mice were transferred to the bioassay laboratory; they were kept on a 12-hour light/dark cycle, 50% relative humidity and $21 \pm 2^\circ\text{C}$. Mice were provided with water and food *ad libitum*. All the bioassays were performed in line with the NIH Guide for the Care and Use of Laboratory Animals; experimental protocols were approved by the Institutional Animal Care and Use Committee.

Effects of dietary BRB, BRB extract, FA, and PA on DBP-induced DNA adducts in the mouse oral tissues

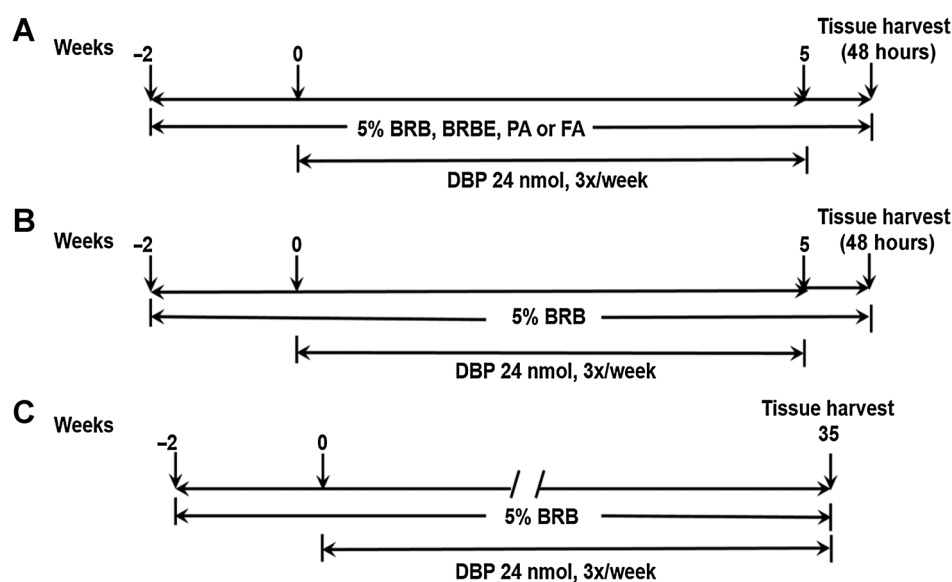
Five groups of mice (6 mice/group) were fed AIN-93 M diet (5% corn oil) as control diet and AIN-93M diet containing BRB (5%); BRB extract (BRBE; 1.6%), anthocyanin enriched fraction extracted from BRB, FA (0.05%, a major phenolic compound in raspberries); and PA (0.2%, a major metabolite of anthocyanins) starting 2 weeks prior to the administration of DBP (24 nmol, three times per week for 5 weeks) by topical application into the oral cavity of mice (Fig. 1A); the various diets were fed until termination of the bioassay.

Several previous preclinical studies indicated that 5% BRB was the optimal level for protection against cancer development in animal models (reviewed in ref. 7). Levels of the other agents were estimated on the basis of the composition of BRB and PA level was based on a published method (26). The dose of DBP was selected on the basis of our previous studies (8, 27). Mice were sacrificed 48 hours after the last dose of DBP and tissues were harvested for DNA isolation.

The analysis of the major deoxyadenosine (dA) adducts derived from DBP by LC/MS-MS was performed using our previously published method (27, 28). Briefly, DNA was isolated from oral tissues using the Qiagen genomic DNA isolation procedure. The level of DNA was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The internal standard [$^{15}\text{N}_5$]anti-*trans*-DBPDE-dA adduct (150 pg) was added to about 60 μg DNA prior to enzymatic hydrolysis. Subsequently, in the presence of 10 μL of 1 mol/L MgCl_2 /mg DNA and DNase 1 (0.2 mg/mg DNA), DNA was hydrolyzed at 37°C for 1.5 hours, followed by overnight incubation of the mixture with snake venom phosphodiesterase (0.08 U/mg DNA) and alkaline phosphatase (2U/mg DNA).

Figure 1.

Experimental protocol for the effects of BRB on DBP-induced (A) DNA damage, (B) DNA methylation, and (C) tumorigenesis in the mouse oral tissues.



The analysis of dA was performed by HPLC using an aliquot of the DNA hydrolysate. Partial purification of the remaining supernatant was achieved using an Oasis HLB column (1 cm³, 30 mg; Waters Ltd.). The analysis of DBP-dA adduct was performed on an API 3200 LC/MS-MS triple quadrupole mass spectrometer interfaced with an Agilent 1200 series HPLC using an Agilent extend-C18 5 micron 4.6 × 150 mm column. Adducts were monitored in multiple reaction monitoring modes and the MS-MS transition of m/z 604 → m/z 335 and m/z 609 → m/z 335 were monitored for targeted adducts and the internal standard to maximize the sensitivity, respectively.

Effects of dietary BRB on DNA methylation in the oral cavity of mice treated with DBP

We found that the inhibition of DBP-DNA adducts formation by dietary 5% BRB was not significantly different from the other dietary groups (cf. Results), and BRB as a whole food approach is easy to obtain at a lower cost. On the basis of these findings, in this study, we tested the hypothesis that dietary 5% BRB may alter DNA methylation induced by DBP in the early stage of carcinogenesis prior to any morphologic abnormalities but at a stage of maximum DNA damage observed in the mouse oral cavity (12). The goal of this study was to investigate the chemopreventive effects of dietary BRB on oral tumorigenesis induced by DBP and to elucidate the mechanisms that may account for the cancer preventive activity of BRB in this animal model. Thus, using a group of mice treated with DBP as a control to compare with those treated with DBP+BRB allowed us to identify the methylation sites altered by BRB in the presence of DBP. Two groups of mice ($n = 3$ per group), after 1 week of quarantine, were fed either control AIN-93M diet or AIN-93M diet containing 5% BRB for 2 weeks prior to topical application of DBP (24 nmol in DMSO) three times a week for 5 weeks (Fig. 1B). Mice were

sacrificed 48 hours after the last dose of DBP. Oral tissues were isolated from the same anatomic sites of mice and pooled together for DNA extraction.

Genomic DNA from oral tissues of mice treated with DBP and fed control diet or a diet containing 5% BRB as described above were extracted and purified according to the DNeasy Blood and Tissue Kit (Qiagen). The DNA was then subjected to enhanced reduced representation bisulfite sequencing (ERRBS) analysis as described previously by our group (12). Briefly, DNA was digested by *MspI* followed by end repair, adenylation, and adapter ligation with a modification of bead size selection to capture *MspI* fragments of 70 to 320 bp size. The resulting libraries were bisulfite-converted followed by PCR amplification and read by 1 × 50 bp on HiSeq and the resulting CASAVA-demultiplexed.fastq files were subjected to downstream analyses. Base calls of bisulfite-treated sequencing reads were mapped to the mm9 mouse assembly and methylation calls were performed using Bismark v0.10.1 (Babraham Bioinformatics). The differential methylation between DBP-BRB versus DBP was calculated using methylKit v0.9.2 R package with “normalizeCoverage” function to avoid bias introduced by systematically more sequenced samples. Differentially methylated bases with q -value <0.01 and percent methylation difference >25% were extracted. The methylKit only analyzes bases covered in all samples and takes into account samples size to calculate both P - and q -values. In particular, methylKit accepts either a single sample per group in which case it uses the Fisher exact test to calculate P values or multiple samples per group in which case it uses logistic regression. These differentially methylated sites (DMS) were annotated with genic parts information from the UCSC Table Browser (mm9 refGene table). Ingenuity Pathways version 2014-10-25 was then performed on the genes with altered methylation patterns to obtain significant canonical pathways associated with dietary BRB.

The effect of BRB on DBP-induced tumorigenesis in the mouse oral tissues

The design of this protocol consisted of two groups of mice (30/group) at the age of 8 weeks. Mice were treated (by topical application into the oral cavity) with DBP (24 nmol) that had been dissolved in DMSO three times per week for 35 weeks (Fig. 1C). In group 1, mice were fed AIN-93M control diet and those in group 2 were fed AIN-93M diet containing 5% BRB. Feeding started 2 weeks before DBP administration and continued until termination of the bioassay. In our previous study (19), no histologic abnormalities were observed in the oral cavity of mice treated with the vehicle (DMSO) by topical application and fed AIN-93M diet containing 5% BRB, and thus such a group of mice was not included in this study. During the progress of the bioassay, mice were weighed weekly in the first month and then every 2 weeks until termination. Mice were culled from each group and sacrificed by CO₂ asphyxiation if a sudden weight loss (>20%) or tumors size over 0.5 cm in diameter were observed. At sacrifice, we collected soft tissues of the oral cavity which included tongue, pharynx, and other oral tissues (hard palate, buccal mucosa, and floor of the mouth). Tissues were fixed in 10% neutral-buffered formalin and then processed in an automated Tissue-Tek VIP processor and paraffin-embedded with a Tissue-Tek TEC embedding station. Staining with hematoxylin and eosin were applied to sections that were cut at 6 μm. A board-certified veterinary pathologist (HA) blinded to treatment protocol examined all tissues and lesions received and provided diagnoses based on established International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) for Lesions in Rats and Mice (29, 30).

Results

Effects of BRB powder and related agents on DBP-induced DNA adducts in oral tissues of mice

The goal of this short-term animal bioassay was to rank the potency of BRB and related agents to inhibit DNA damage induced by DBP, a prerequisite step in the multistep carcinogenesis process. Here we showed that BRB (5%), BRBE (1.6%), PA (0.2%), or FA (0.05%) in the diet, starting 2 weeks before carcinogen treatment and continued until termination of the bioassay significantly ($P < 0.05$) reduced the levels of (-)-anti-trans-DBPDE-dA in mouse oral tissues as shown in Fig. 2; these treatments significantly resulted in 19.2%, 29.6%, 23.0%, and 25.3% reduction of the level of (-)-anti-trans-DBPDE-dA in murine oral tissues, respectively. The percent of inhibition was comparable following the various interventions; therefore we selected, for practical purposes, only 5% BRB as the agent in the subsequent bioassays.

Effect of BRB on DBP-induced DNA methylation in the mouse oral tissues

To examine if dietary BRB may alter the DNA methylation induced by DBP, we isolated DNA from histologically normal

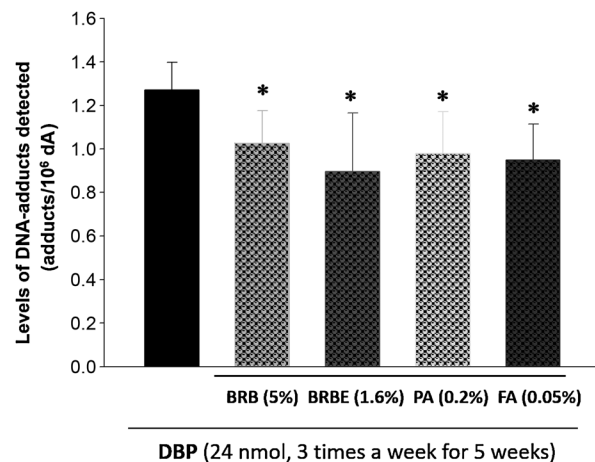


Figure 2.

The effects of BRB (5%), BRBE (1.6%), PA (0.2%), or FA (0.05%) in the diet on the levels of (-)-anti-trans-DBPDE-dA in mouse oral tissues (*, $P < 0.05$).

oral tissues of mice treated with multiple doses of DBP and fed with a control diet or diet containing 5% BRB powder and performed ERRBS analysis coupled with next-generation sequencing. This approach provides a quantitative, single-nucleotide resolution on the status of DNA methylation both within and outside CpG islands. Our data showed that an average of 8.8 million Illumina sequencing reads was generated per sample and the percentages of methylated C in CpG context were 35.2% and 32.6% for DBP and DBP+BRB, respectively. Approximately 72% of the sequencing reads were mapped to either strand of the mouse genome (mm9). These sequencing data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (Accession No: GSE8991).

Comparative methylation analysis was conducted between DBP+BRB versus DBP groups to identify differentially DNA methylated sites (DMS) using methylKit (version 0.9.2) R package (31). Our results showed that about 32% DMS are located in promoters, 18% in exons, 23% in introns, and 26% in intergenic regions (Supplementary Fig. S1A); both unsupervised analyses of hierarchical clustering (1-Pearson correlation distance + Ward clustering method) and principal component analysis (PCA) were performed to examine the correlations among all of these samples as shown in Supplementary Figs S1B and S1C, respectively. Because of limited sample size, no distinct clusterings were observed; however, large variations were noted from DBP+BRB-treated samples in PC1; in PC2, DBP-treated samples showed larger variations as compared with DBP+BRB-treated. The percentage of hyper and hypomethylated differential methylation sites (DMS) out of all covered CpGs for each chromosome (Chr) is shown in Supplementary Fig. S1D.

A total of 960 DMS were identified between DBP versus DBP+BRB with q -value < 0.01 and percent methylation difference $> 25\%$; among them 479 were hypermethylated and 481 were hypomethylated as listed in Supplementary Tables S1A and

S1B, respectively. Among 960 DMS, we found four DMS mapped to genes *Fgf3*, *Qrich2*, *Rmdn2*, and *Cbarp* were hypermethylated by dietary BRB but these loci were hypomethylated by DBP either at the exact position or at proximal site (12). On the other hand, DMS mapped to genes *Vamp3*, *Ppp1r13l*, *Pkm*, and *Zfp316* were hypomethylated by BRB but they were hypermethylated by DBP at proximal sites. Although DBP induced two hypermethylated sites mapped to *Disc1*, BRB induced both hyper- and hypomethylation at proximal sites (Table 1).

Ingenuity pathway analysis (IPA) for the annotated genes identified the top canonical pathways [$-\log(P) \geq 1.3$] as glutamate receptor signaling, IGF-1 signaling, relaxin signaling, molybdenum cofactor biosynthesis, regulation of EMT pathway, and glycolysis I (Supplementary Table S2). It is noted that in addition to *Fgf3*, 2 DMS mapped to genes *Fgf4* and *Fgf13* were also hypermethylated by BRB treatment; these fibroblast growth factors are involved in the EMT pathway.

Effect of BRB on DBP-induced oral tumorigenesis

The inhibitory effects of BRB on DNA damage induced by DBP combined with its effects on epigenetic alterations induced by this carcinogen in the mouse oral cavity provided a strong rationale to further examine the chemopreventive efficacy of BRB against DBP induced oral cancer in the same mouse model as described below.

Body Weights (mean \pm SE) of mice treated with DBP and fed control diet containing 5% BRB, and those treated with DBP and fed control diet are provided in Fig. 3A. The body weights of mice in both groups were increased gradually in a similar pattern. The cumulative mortality of mice during the progress of the bioassay is shown in Fig. 3B. The probability of survival is displayed using a Kaplan–Meier plot with death as an endpoint. A log-rank test was used to evaluate the difference between these groups and no significant difference was observed in survival analysis.

The tumor incidence [including papilloma, carcinoma in situ (CIS) and SCC] in the oral cavity of mice treated with DBP and fed diet containing 5% BRB was significantly ($P < 0.05$)

reduced from 70% to 46.7% (Table 2; Fig. 4), and the incidence of papilloma was also significantly ($P < 0.05$) reduced from 33.3% to 13.3%. SCC in the oral mucosa was reduced from 40% to 33.3%, and CIS was reduced from 13.3% to 3.3%. The overall histological findings in mice treated with DBP and DBP+BRB in the lip, oral cavity, and tongue were summarized in Table S3 and showed in Fig S2; BRB had no protective effect in tumors developed in the lip; only one animal treated either with DBP or DBP+BRB developed SCC.

Discussion

Genotoxicity (DNA damage, genetic mutations, chromosomal abnormalities) induced by chemicals found in the human environment has been shown to play an essential step in the multistep carcinogenesis process (32–34). Our first goal of this study was to rank in a short-term *in vivo* study, the efficacy of BRB, some of its constituents, and a major metabolite (PA) derived from a major and a biologically active component of BRB (anthocyanins) on DBP-induced DNA damage in the mouse oral cavity. PA accounts for about 70% of the metabolites of anthocyanins in humans (35) and is an inhibitor of chemically-induced cancers in rodents (36). FA, a phenolic acid, is also present in raspberries and can account, in part, for their chemopreventive activities (37). We demonstrated for the first time using LC/MS-MS method that BRB and related agents have comparable but significantly inhibited the levels of (-)-*anti-trans*-DB[a,l]PDE-dA in the oral tissues of mice treated with DBP. These *in vivo* findings are consistent with our previous *in vitro* studies (28, 38), demonstrating that anthocyanin components of BRB and its metabolite, PA, are in part responsible for the inhibitory effects of BRB on the DBP-induced DNA adducts formation. Our results also support that dietary consumption of BRB and its related components can inhibit the metabolic activation of DBP or enhance the detoxification of DB[a,l]PDE and the DNA repair efficacy in mouse oral cavity; collectively, these effects of BRB may account for the inhibition of the subsequent mutagenesis

Table 1. Comparisons of differentially methylated sites identified in both ERRBS of DBP-BRB vs. DBP and DBP vs. DMSO.

Chr	Gene	DBP-BRB vs. DBP		DBP vs. DMSO	
		Methylation differences	Position	Methylation differences	Position
chr7	<i>Fgf3</i>	34.18234	152014445	-37.6374	152014445
chr11	<i>Qrich2</i>	28.62254	116317982	-36.7897	116317982
chr17	<i>Rmdn2</i>	35.05828	80062330	-28.5125	80062330
chr10	<i>Cbarp</i>	34.17839	79594923	-27.812	79594761
chr4	<i>Vamp3</i>	-26.8916	150809404	38.833	150503856
chr7	<i>Ppp1r13l</i>	-32.7257	19955765	43.2683	19955390
chr9	<i>Pkm</i>	-29.0348	59503922	43.3803	59504569
chr5	<i>Zfp316</i>	-29.0043	144014872	25.2916	144049680
		-28.8456	144014905		
chr8	<i>Disc1</i>	-28.7854	127736702	36.25	127736689
		27.96859	127615449	35.3156	127736699

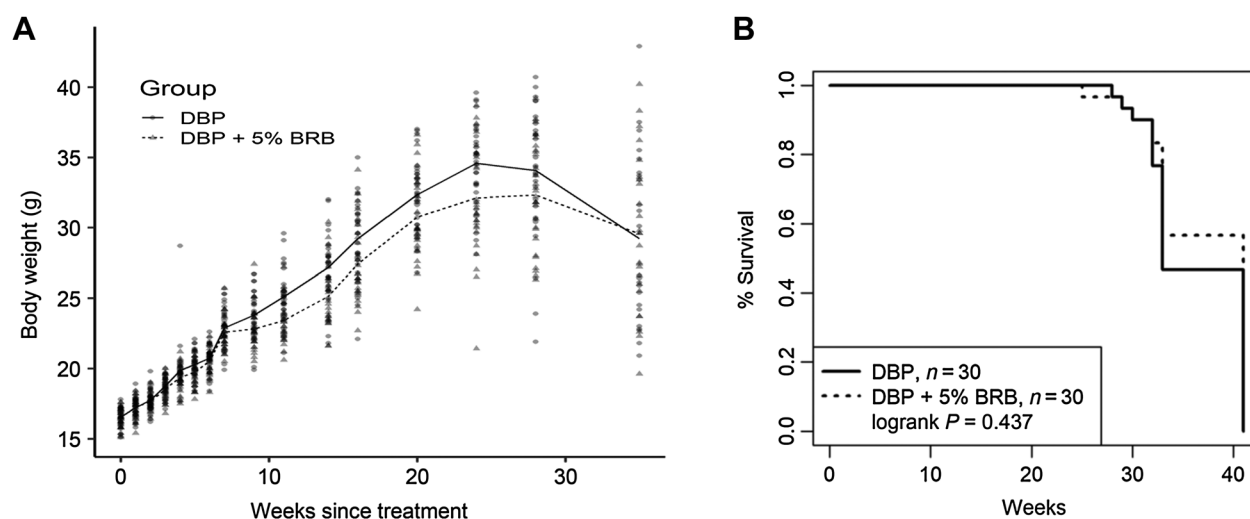


Figure 3.

Body weights (**A**) and percentage survival (**B**) of B6C3F1 mice treated by topical application of DBP (24 nmol, three times a week) and DBP + 5% BRB during the progress of the bioassay.

and carcinogenesis resulting from exposure to DBP. Although anthocyanins are the most abundant compounds in BRB and can account for much of their antioxidant activity (39, 40), they are expensive for routine feeding studies. Considering the cost and easy access, 5% BRB in the diet was used for the remaining mouse bioassays performed in this study.

In addition to genotoxicity, emerging evidence strongly indicates that epigenetic alterations can also play a critical role in the initiation and progression of environmental carcinogenesis (41, 42). Many types of DNA damage (covalent DNA adducts, oxidative lesion, abasic sites, photodimers, etc.) have been shown to alter DNA methylation via various mechanisms associated with the formation of DNA lesions and/or by alteration of DNA methyltransferases (12, 43). Previously, we reported that DBP was able to alter DNA methylation profile in the mouse oral cavity at a stage prior to any morphologic abnormalities; we also identified that *Fgf3*, a gene frequently amplified in HNSCC, was hypomethylated by DBP and may serve as a potential biomarker for early detection of oral

carcinogenesis (12). Given the reversibility, although not all of the epigenetic changes, we examined for the first time if BRB may modulate DNA methylation in a manner consistent with prevention of oral carcinogenesis induced by DBP. The effect of BRB on DNA methyltransferase 1 (DNMT1) has been reported by others (44), indicating the BRB can decrease the protein expressions of DNMT1. In our previous study (12), we reported that changes in mRNA expressions of DNMT1, DNMT3a and DNMT3b induced by DBP were observed, but not significant; however, we did not determine the effect of DBP on the activities of DNA methyltransferases. Consistent with the effect of BRB on DNMT1 (44), we noted that dietary BRB reduced the percentages of methylated C in CpG context from 35.2% to 32.6%. Induction of genomic hypomethylation has also been shown to preferentially inhibit the development of squamous cell carcinoma in the tongue and esophagus induced by the synthetic 4-nitroquinoline-1-oxide in a mouse model (45).

Table 2. Inhibition of tumorigenesis by BRB in the oral cavity of DBP-treated mice.

	Treatments	
	DBP	DBP + BRB
Number of mice	30	30
CIS	4 (13.3) ^a	1 (3.3)
Papilloma	10 (33.3) ^a	4 (13.3) ^c
SCC	12 (40.0) ^a	10 (33.3)
Total tumors	21 (70.0) ^b	14 (46.7) [*]

^aNumber in parentheses, percentage of mice developed tumors.

^bTumors induced by DBP in the oral cavity consist of papilloma, CIS and SCC.

^cTumor incidence was significantly reduced compared with DBP treatment (*, $P < 0.05$).

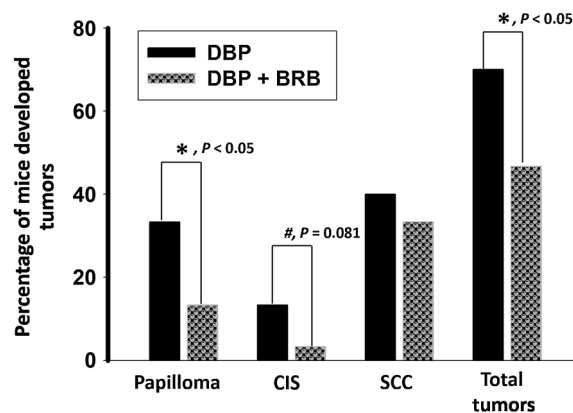


Figure 4.

5% BRB inhibits DBP-induced tumorigenesis in the oral cavity of mice (*, $P < 0.05$).

In light of the effects of BRB on aberrant DNA methylation induced by DBP, in this study we conducted a genome-wide DNA methylation and identified 960 differentially methylated sites as well as the top canonical pathways including EMT that may account for the epigenetic effect of BRB. DNA methylation is one of the epigenetic mechanisms that may regulate gene expression. Many aberrant DNA methylation occurred in the early stage of carcinogenesis without changes in gene/protein expression; thus, methylation changes can be a powerful biomarker for early detection (12) as well as for monitoring the effects of chemopreventive agents. Several DMS that were induced by DBP treatment were found to be altered by dietary BRB; these DMS were mapped to genes including *Fgf3*, *Qrich2*, *Rmdn2*, *Cbarp*, *Vamp3*, *Ppp1r13l*, and *Pkm*. It is noted that BRB induced hypermethylation of *Fgf3* at the same position that was hypomethylated by DBP. In addition to *Fgf3*, we also found 2 hypermethylated sites mapped to genes *Fgf4* and *Fgf13* at promoter region. Although BRB induced hypermethylation of *Fgf4* and *Fgf13* located in the promoter region, hypermethylation of *Fgf3* was located at 10 kb upstream of the *Fgf3* transcription start site and is not within a CpG island. All these three fibroblast growth factors were involved in the regulation of EMT pathway, suggesting that BRB may prevent DBP-induced oral carcinogenesis through epigenetic modulation of genes involved in the EMT pathway. In fact, the reversal of the EMT pathway can account for the inhibition of cancer invasion in a preclinical lung cancer models (46). Encouraged by the results of the effects of BRB on genetic (DNA damage) and epigenetic (DNA methylation) modulations, the third goal of this study was to examine the effect of BRB on DBP-induced oral tumorigenesis in a long-term bioassay in mice.

Our results demonstrated that mice tolerated 5% BRB in the diet based on survival and body weights. Furthermore, our current finding is in line with our previous report demonstrating the protective effect of BRB against the ultimate and direct-acting carcinogenic metabolite (DB[a,l]PDE) of DBP. The modulating effects of BRB on phase I and phase II drug metabolism enzymes and DNA repair enzymes can account for the inhibition of DNA damage induced by DBP and its metabolite DB[a,l]PDE (7). Similar to our previous finding using DB[a,l]PDE, a significant protective effect of BRB was observed in benign tumors, but the inhibition of malignant formation (CIS, SCC) did not reach significance. We believe that significant inhibition of malignancy can also be reached by extending the duration of the bioassay to provide an ample opportunity for benign tumors to transition to malignant tumors; unfortunately, the bioassay was not extended because the majority of the tumors' size were >0.5cm that hinder eating and drinking and thus the animals had to be sacrificed. In contrast to DB[a,l]PDE, which induced both oral and tongue tumors, DBP induced primarily oral tumor and thus the inhibitory effect of BRB on tongue tumors cannot be assessed (only one animal developed SCC in the tongue induced by DBP or DBP + BRB). Literature data demonstrate that preferential inhibition of tumorigenesis in target organs which come in contact with BRB (7). Exposure of the mouse lip to BRB may

be extremely short as compared with the oral cavity within which BRB may retain longer and such differential contact of the two sites may, in part, explain the lack of protection on DBP-induced tumors in the lip.

As discussed above, we showed that dietary 5% BRB inhibits DBP-induced oral tumorigenesis through mechanisms including inhibition of DNA adduct formation and alteration of DNA methylation induced by DBP. In our long-term efficacy study, because BRB was fed before carcinogen treatment and continued until termination, we could not distinguish the effect of BRB on both phases of carcinogenesis (initiation vs. promotion/progression). However, the inhibitory effects of BRB on DNA damage observed in this study support its protective role during the initiation phase of carcinogenesis. It is also important to acknowledge additional limitations in this study. Only female mice were used, and thus our results cannot provide insights on sex differences regarding the higher incidence of oral cancer in men than in women (47). Concerning DNA adducts, we focused only on the detection and quantification of the major dA adducts derived from DBP. However, as reported by us recently, the minor dG adducts could be critical in the induction of mutagenesis induced by DBP *in vivo* (48). Considering its safe usage and ease of administration, combined with its effects on modulating markers critical in carcinogenesis in both preclinical and clinical studies, future studies should be aimed at determining the effect of BRB on the initiation stage of carcinogenesis by assessing levels of DNA adducts derived from select tobacco carcinogens in smokers which are at high risk of developing oral cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: K.-M. Chen, Y.-W. Sun, S. Amin, K. El-Bayoumy
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