Bitter Melon Prevents the Development of 4-NQO-Induced Oral Squamous Cell Carcinoma in an Immunocompetent Mouse Model by Modulating Immune Signaling

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, and tobacco is one of the most common factors for HNSCC of the oral cavity. We have previously observed that bitter melon (Momordica charantia) extract (BME) exerts antiproliferative activity against several cancers including HNSCC. In this study, we investigated the preventive role of BME in 4-nitroquinoline 1-oxide (4-NQO) carcinogen-induced HNSCC. We observed that BME feeding significantly reduced the incidence of 4-NQO-induced oral cancer in a mouse model. Histologic analysis suggested control 4-NQO-treated mouse tongues showed neoplastic changes ranging from moderate dysplasia to invasive squamous cell carcinoma, whereas no significant dysplasia was observed in the BME-fed mouse tongues. We also examined the global transcriptome changes in normal versus carcinogen-induced tongue cancer tissues, and following BME feeding. Gene ontology and pathway analyses revealed a signature of biological processes including “immune system process” that is significantly dysregulated in 4-NQO-induced oral cancer. We identified elevated expression of proinflammatory genes, s100a9, IL23a, IL1β and immune checkpoint gene PDCD1/PD1, during oral cancer development. Interestingly, BME treatment significantly reduced their expression. Enhancement of MMP9 (“ossification” pathway) was noted during carcinogenesis, which was reduced in BME-fed mouse tongue tissues. Our study demonstrates the preventive effect of BME in 4-NQO-induced carcinogenesis. Identification of pathways involved in carcinogen-induced oral cancer provides useful information for prevention strategies. Together, our data strongly suggest the potential clinical benefits of BME as a chemopreventive agent in the control or delay of carcinogen-induced HNSCC development and progression.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) represents heterogeneous disease, and oral cavity squamous cell carcinomas including tongue cancers are more common. Oral cancer is often associated with tobacco use, alcohol consumption, an unhealthy diet, an inactive lifestyle and poor oral hygiene. The overall survival rate has not improved in the past several of decades, despite significant improvements in surgical procedures, radiotherapy, and chemotherapy, and several factors that contribute to this poor outcome. Thus, there is unmet need for prevention and additional therapeutic intervention.

The 4-NQO (4-nitroquinoline 1-oxide) oral cancer model allows us to study the initiation and prevention of chemically induced cancers of the oral epithelium in vivo (1, 2). In this model, immunocompetent mice treated with 4-NQO develop invasive squamous cell carcinoma of the oral cavity with near 100% penetrance. (3). In this model, immunocompetent mice treated with 4-NQO develop invasive squamous cell carcinoma of the oral cavity with near 100% penetrance. This model mimics how chronic tobacco abuse contributes to human oral cancers and therapeutic treatments can reduce or prevent these malignancies (3). 4-NQO is a synthetic, water-soluble carcinogen, which mimics the chronic tobacco consumption effect by promoting DNA adduct formation, A-G nucleotide substitution, and intracellular oxidative stress, resulting in histologic and molecular alterations similar to human oral carcinogenesis (3).

One of the main challenges in cancer therapy is the excessive toxicity of chemotherapeutics due to their
nonselective activity. Natural products play a critical role in the discovery and development of numerous drugs for the treatment of various types of deadly diseases, including cancer. Therefore, the use of natural products as preventive medicines is becoming increasingly important. In our previous studies, we showed that bitter melon (Momordica charantia) extract (BME) treatment of human cancer cells induces cell-cycle arrest by altering critical signaling molecules and impairing cell growth (4–6). BME feeding also prevented high-grade prostatic intraepithelial neoplasia formation in the TRAMP mouse model (5). We recently observed that BME augments NK cell–mediated HNSCC killing activity and reduces the Th17 cell population in the tumor, implicating an immunomodulatory role in a syngeneic mouse model (7, 8).

In this study, we generated oral/tongue squamous cell carcinoma (OSCC) mouse model by adding 4-NQO to drinking water. To examine the effect of BME in the prevention of OSCC development, we included a group of mice with 4-NQO with BME to drinking water. Our results strongly demonstrated that BME feeding reduced 4-NQO-induced OSCC growth. Our results also provide important information about the molecular features of the carcinogenesis and chemoprevention. To our knowledge, this is the first study describing the prevention of carcinogen-induced oral cancer progression by BME in immunocompetent mouse model.

Materials and Methods

Tumor development in the mouse oral cavity

C57BL/6 mice (average body weight 18 gm) were obtained from Charles River Laboratories and housed in a specific pathogen-free facility at the Saint Louis University (St. Louis, MO). Mice were maintained at 25°C ± 5°C temperature, with alternating 12-hour light/dark cycle and 45% to 55% humid conditions. All the animal experiments were carried out in accordance with NIH (Bethesda, MD) guidelines, following a protocol approved by the Institutional Animal Care and Use Committee of Saint Louis University.

For oral cancer development, mice (n = 10) were given 4-NQO (50 μg/mL) in their drinking water for 16 weeks, then only water until the end of the experiment. The other group of mice (n = 10) received BME (30% v/v, 600 mg/mouse) in the drinking water along with 4-NQO treatment for 16 weeks, and then continued with only BME until the end of the experiment. The BME was prepared from the Chinese variety of young bitter melons (raw and green) as described previously (7). Briefly, BME was extracted from bitter melon without seeds using a household juicer, centrifuged at 15,000 × g at 4°C for 30 minutes, and stored at −80°C. The dose of the BME for animal experimentation was selected on the basis of our previous studies. Experimental design is summarized in Fig. 1A. Mice were sacrificed at 22 weeks, tongue tissue was macroscopically examined, and the number of macroscopic lesions was counted. One part of tissue was fixed in formalin for histopathologic analysis, and the other part was snap frozen in liquid nitrogen for biochemical analysis.

Histopathologic analysis

Histologic evaluation was performed with formalin-fixed, paraffin-embedded tongue tissues. The tongue sections were stained by hematoxylin and eosin (H&E) and observed under brightfield microscopy. Histopathologic stages were confirmed by a pathologist in a blinded fashion.

RNA isolation and RNA-Seq analysis

Total tissue RNA was extracted from frozen tissue samples by TRIzol reagent and subjected to Next Generation RNA Sequencing (IlluminaNextSeq 500 1x75) at MQgene, LLC. TopHat and Bowtie software installed in Illumina BaseSpace was used for assembling contigs. The CLC Genomics Workbench 10.0 software was used for additional data analysis. The reads per kilo base of exon model per million mapped reads (RPKM) were calculated, and statistical analysis was performed to determine differential gene expression among different groups. Gene Ontology (GO) mapping was performed using custom scripts. The mapping between reads (ENSEMBL identifiers) and GO identifier codes was downloaded from EBI. GO enrichment was assessed using Fisher exact P value using a contingency table of the number of genes that observed to be differentially expressed with a GO category, the total number of transcripts in the GO category, the total number of differentially expressed genes, and the total number of GO categories in the RNA sequencing (RNA-Seq) dataset.

mRNA expression analysis

Total RNA (1 μg) was used with SuperScript III Reverse Transcriptase (Life Technologies) following the manufacturer’s protocol for cDNA synthesis. Gene expression of S100a9 (Mm 00656925_m1), IL23a (Mim 00518984_m1), IL1b (Mm 00434228_m1), PDCD1 (Mm 01285676_m1), and MMP9 (Mm 00442991_m1) was carried out by real-time PCR using the TaqMan gene expression assay (Thermo Fisher Scientific). The mouse 18s gene was used as an endogenous control and for target gene normalization. The relative gene expression was analyzed by using the 2−ΔΔCt method, and relative expression was graphically presented.

Protein extraction and Western blot analysis

Tissue lysates were prepared from the frozen samples by using 2 × SDS sample buffer, and Western blot analysis was performed using a specific antibody to proliferating cell nuclear antigen (PCNA; 1:1,000, Santa Cruz Biotechnology). The blot was reprobed with GAPDH antibody (Cell Signaling Technology) to compare protein load in each
lane. Densitometric analysis was done by using ImageJ software.

**Statistical analysis**

Data obtained from the 4-NQO–treated (cancer) group were compared with normal mouse tongue (as control), and data obtained from 4-NQO + BME-fed (experimental) group were compared with cancer group. Statistical analysis was performed using the Student t test. P < 0.05 was considered as statistically significant. Data were expressed as mean with SD.

**Results**

**Effect of BME on 4-NQO–induced mouse oral cancer**

Our previous studies demonstrated antiproliferative and immunomodulatory effects of BME on in vitro and in vivo oral cancer models (6–8). In this study, we wanted to examine the preventive effect of BME in 4-NQO–induced cancer model, which mimics the tobacco-related oral cancer. For this, mice (C57BL/6) were given 4-NQO with or without BME in the drinking water. The volume of water intake and animal health were closely monitored. We terminated our experiment at the 22nd week, as 2 mice in 4-NQO–treated group had large tumor in the tongue and looked sick, were not eating well, and had lost significant weight. Tongue was harvested from each mouse for histologic and biochemical analysis. The body weight of the mice was lower in 4-NQO–treated group as compared with BME-fed group after 18 weeks (Fig. 1B). Macroscopic lesions were more prominent in 4-NQO–treated group as compared with BME-fed group after 18 weeks (Fig. 1B). Macroscopic lesions were more prominent in 4-NQO–treated group as compared with BME-fed group (Fig. 1C). Tongue histology was analyzed by a pathologist (K. Schwetye) in a blinded fashion. The number of lesions per mouse was counted, and based on histopathologic examination classified as mild to severe dysplasia, squamous cell carcinoma, hyperkeratosis, acantholysis, large and abnormal nuclei, and tumor-infiltrating lymphocytes (TIL) were observed in the 4-NQO–treated group. Two mice developed invasive squamous cell carcinoma, while most of the mice displayed severe dysplasia. Interestingly, no remarkable histologic abnormalities were seen in BME-fed mice, and only...
2 mice developed mild dysplasia. Representative histologic images are shown in Fig. 2A. We also examined the expression of PCNA in both groups of mouse tongue. Western blot analysis demonstrated that PCNA expression was inhibited in BME-fed mice as compared with 4-NQO-treated group (Fig. 2B).

RNA-Seq analysis of 4-NQO-induced oral cancer and BME treatment

To understand the global gene expression pattern, RNA-Seq was performed from RNA of normal mice (control-without any treatment), 4-NQO-treated mice (cancer group), and mice treated with 4-NQO + BME (BME-fed experimental group) tongue tissues in biological triplicates. The RNA sequence data generated a set of 22,782 transcripts. The differentially expressed genes were obtained from TopHat/Cufflinks analysis (specifically CuffDiff) as described earlier (9). A P value cutoff of 0.01 was used, to account for expression values across all replicates. Out of annotated 22,782 transcripts (data not shown), differential expression between normal versus 4-NQO-induced tumor, and 4-NQO-induced tumor versus BME-fed experimental group was shown (Fig. 3A and B). Among them, 6,242 genes were differentially expressed between the 4-NQO-treated and normal (control) group, of which 2,146 genes were unique (Fig. 3C). On the other hand, 4,482 genes were differentially regulated in the experimental (BME and 4-NQO-fed group) versus 4-NQO-treated group, where 634 genes are unique (Fig. 3C). Furthermore, 1,330 genes were differentially regulated between the experimental and normal (control) group and 466 genes were unique. There were 386 genes commonly altered among the three groups (Fig. 3C). Thus, the data indicate modulation in expression of a huge number of genes during BME-mediated tongue cancer prevention.

GO analysis in 4-NQO-induced oral cancer and BME-fed mice

Next, GO analysis was performed using custom scripts (see Materials and Methods for details) as described previously (10). GO analysis showed significantly (P < 0.05) upregulated GO categories including “keratin filament,” “ion transport,” “structural molecule activity,” “membrane,” “calmodulin binding,” and “regulation of ion transmembrane transport” in the 4-NQO-induced tongue samples as compared with normal group (Fig. 4A). In contrast, “inflammatory response,” “extracellular space,” “cytokine activity,” “immune response,” “structural molecule activity,” and “cell chemotaxis” were significantly downregulated in the 4-NQO-induced cancer group as compared with normal samples (Fig. 4A). BME treatment
resulted in significant upregulation of "extracellular space," "cytokine activity," "immune response," "inflammatory response," "cell chemotaxis," and "positive regulation of apoptotic process" (Fig. 4B), and downregulation of "keratin filament," "extracellular region," "GTP binding," and "lipid metabolic process" in the experimental group as compared with the 4-NQO–induced cancer group (Fig. 4B).

The GO categories, for which deregulated genes were significantly enriched between experimental and cancer groups, were selected. The differentially expressed genes between the groups were categorized under GO of "biological processes" (Fig. 5). For both comparisons, significantly enriched ($P = 2.2 \times 10^{-16}$) GO categories were "signal transduction (GO:0007165)," "apoptosis process (GO: 0006915)," "metabolic process (GO:0008152)," "cell adhesion (GO:0007155)," "lipid metabolism (GO:0006629)," "immune system process (GO: 0002376)," "angiogenesis (GO: 0001525)," "ossification (GO: 0001503)," and "G1/S transition of mitotic cell cycle (GO: 0000082)" (Fig. 5).

**Comparison of individual transcripts in 4-NQO–induced oral cancer and BME treatment from immune system process**

We have shown previously that BME treatment modulates signal transduction pathways and induces apoptotic cells death (6), and current RNA-Seq data are in agreement with our previous results. We also recently observed immunomodulation by BME in a syngeneic HNSCC mouse model. Furthermore, HNSCC has been intensively studied as an immunosuppressive disease (11), and we focused our analysis here in immune modulation. We compared the mRNA expression change of selected individual genes of the significantly enriched immune system processes between the 4-NQO–induced cancer group and BME-fed experimental group. RNA-Seq data in this study suggested a significant upregulation of s100a9 (686.7-fold), IL23a...
(13.41-fold), IL1b (18.88-fold), and PDCD1 (17.61-fold) in the 4-NQO–induced cancer group as compared with normal mice under "immune system process (GO: 0002376). Elevated expression of proinflammatory molecules s100a9, IL23a, and IL1b has been reported in different cancers, including human oral cancer (12–15). Increased expression of immune checkpoint regulatory gene PDCD1 has also been reported in human oral cancer and other cancers (16). Interestingly, the BME-fed group displayed 3.02-, 7.57-, 5.99-, and 1.7-fold downregulation of s100a9, IL23a, IL1b, and PD-1 genes, respectively. For validation, we examined expression status of these transcripts in 4-NQO–induced tongue tissues with and without BME treatment. Similar to the RNA-Seq data, significant upregulation of s100a9, IL23a, IL1b, and PDCD1 was observed in 4-NQO–induced cancer tongue tissues as

Figure 4.
Gene ontology (GO) analysis of the genes in different groups. A, GO analysis of the genes whose mRNA levels were significantly upregulated or significantly downregulated in 4-NQO-treated cancer group compared with normal. B, GO analysis of the genes whose mRNA levels were significantly upregulated or significantly downregulated in BME-fed experimental group compared with 4-NQO-treated cancer group.
compared with normal tongue tissues (Fig. 6A). This further confirmed that modulation of the immune system might be necessary for progression of in vivo OSCC. We also observed significant downregulation of these genes in BME-fed mice as compared with 4-NQO–induced cancer group (Fig. 6A), indicating an important mechanism of chemoprevention by BME. String analysis showed association of these genes with each other under the immune system processes (Fig. 6B), suggesting functional importance of this biological process.

Under “ossification process” (GO: 0001503), RNA-Seq data showed 6.8-fold upregulation of MMP9 in 4-NQO–induced oral cancer tissues and 8.1-fold downregulation in the BME-fed group as compared with cancer group. Increased MMP9 expression has been reported in human oral cancer (17, 18). A significant upregulation of MMP9 was observed in 4-NQO–induced cancer tissues; MMP9 was also significantly downregulated in BME-fed group (Fig. 7A). String analysis of ossification process shows the potential functional association of MMP9 and other molecules under this biological process (Fig. 7B).

**Discussion**

In this study, we observed that BME feeding in the 4-NQO OSCC mouse model significantly reduced the incidence of tongue tumor as compared with 4-NQO–treated mice without any apparent sign of toxicity. Histologic study revealed that most of the BME-fed mice displayed no histopathologic abnormality. On the other hand, 4-NQO–treated mice displayed a range of neoplastic changes, from moderate dysplasia to invasive squamous cell carcinoma. The identification of molecular targets is important in terms of monitoring the clinical efficacy of cancer therapeutic strategies. PCNA plays a crucial role as an integral component of the eukaryotic DNA replication machinery in normal cellular growth and differentiation (19) and is required for cell growth and cell-cycle progression in mammalian cells. Our data strongly demonstrated a reduced expression of PCNA in tongue tissues of BME-fed mice as compared with carcinogen only–treated mice. In the United States, head and neck cancer accounts for 3% of malignancies, with approximately 63,000 Americans developing head and neck cancer annually and 13,000 dying from the disease (20). Employing chemopreventive strategies will reduce recurrence and/or secondary tumor growth (metastasis) and will have beneficial effect clinically to improve patients’ survival with HNSCC. Our pilot study using limited number of mice suggested that BME feeding with 4-NQO delayed tumor initiation (~11th week of treatment) in mouse tongue (data not shown), suggesting the chemopreventive implication of BME.

We have shown previously that BME treatment in HNSCC inhibited c-Met–phosphoStat3 signaling pathway (6). Targeting Stat3 signaling by static in 4-NQO–induced HNSCC model has been reported earlier (21). Recently, sulforaphane treatment in the same model showed...
Figure 6.
Validation of some gene expression involved in "immune system process (GO: 0002376)" during 4-NQO–induced tongue carcinogenesis and prevention by BME. A, Relative mRNA expression of s100a9, IL23a, IL1b, and PDCD1 of different groups was analyzed by quantitative RT-PCR. Mouse 18S gene was used as endogenous control and for target gene normalization. Data are represented as mean ± SD. Small bar, SE (*, P < 0.05). B, String analysis network module showed functional association of differentially expressed genes under GO of "immune system process." Sky blue lines, known interaction from curated databases; pink lines, experimentally determined; green lines, predicted interaction of gene neighborhood; red lines, gene fusion; blue lines, gene cooccurrence; colored nodes, query proteins and first shell of interactors; white nodes, second shell of interactors.
encouraging data of reduced HNSCC by targeting Stat3 targeting (22). Intraperitoneal injection of metformin, a common diabetes drug, also prevented the development of HNSCC (23). Crude extract of bitter melon displayed anticancer effect in several preclinical models with different mechanism (5, 6, 24, 25). There are several components identified as active compounds for BME with limited follow-up studies. We identified cholesteryl β-D-glucopyranoside as an active component of BME in in vitro study (unpublished data). However, efficacy of this compound is not very strong in our in vitro systems as compared with whole BME. In fact, much of the evidence in cancer chemoprevention suggests that bitter melon crude extract (mimicking the whole fruit components) has a stronger effect as compared with fractionated active components. A similar result was reported from blackberry extract (26). Furthermore, use of whole plants or their simple extracts is cost-effective and less toxic. Therefore, BME as a chemopreventive agent in controlling carcinogen such as tobacco-induced oral cancer is appealing.

Global transcriptome analysis is important to understand the molecular mechanism of carcinogenesis and chemoprevention. Limited information about tobacco-related HNSCC transcriptomes was available, and their modulation following BME feeding remains unknown. We observed differential expression of transcripts in the normal and 4-NQO–induced cancer groups. We also observed alternation of transcripts with BME-fed mice in comparison with the experimental group. We recently reported that BME exerts an immunomodulatory role in an HNSCC syngeneic mouse model (7, 8). In our RNA-

Figure 7. Validation of gene expression involved in “ossification (GO: 0001503)” during 4-NQO–induced oral carcinogenesis and prevention by BME. Relative mRNA expression of MMP9 in different groups was analyzed by quantitative RT-PCR. The mouse 18S gene was used as endogenous control and for target gene normalization. Data are represented as mean ± SD. Small bar indicates SE (*, P < 0.05). B, String analysis showed possible functional association of MMP9 and other molecules under GO of “ossification process.” Sky blue lines, known interaction from curated databases; pink lines, experimentally determined; green lines, predicted interaction of gene neighborhood; red lines, gene fusion; blue lines, gene cooccurrence. Colored nodes, query proteins and first shell of interactors; white nodes, second shell of interactors.
Seq data, we observed modulation in “immune response” and “inflammatory response,” in the BME-fed mice. The inflammatory mediators such as cytokine/chemokines present in the tumor microenvironment either promote or inhibit inflammation-mediated tumorigenesis depending on the immune defense mechanism (27). Different NSAIDs showed beneficial effects in combination with conventional therapies in the treatment of different cancers (28). Thus, modulation of these pathways may be an important chemoprevention mechanism by BME with respect to oral cancer.

Immunosuppression was noted in HNSCC. We identified important molecules in the “immune system process” from our array data and validated the expression of the molecules in 4-NQO–induced and BME-fed tongue tissues. We observed significant upregulation of s100a9, IL23α, and IL1β transcripts in 4-NQO–induced cancer tissues. Elevated expression of proinflammatory markers s100a9, IL23α, and IL1β in the cancer samples indicated their importance in in vivo oral carcinogenesis, as reported in human cancers including HNSCC (12–14). However, downregulation of s100a9 was noted recently in oral cancer samples (29). It is possible that s100a9 may have anti- or protumor responses depending on the intracellular milieu. Elevated expression of s100a9 was reported earlier in the 4-NQO–induced mouse tongue cancer study (30), in agreement with our observation. Furthermore, pharmaceutical targeting of s100a9 showed an effective result in phase II clinical trial against metastatic prostate cancer (31).

IL23 is important for the differentiation of Th17 lymphocytes, and its expression depends on tumor microenvironment. We have shown previously that Th17 cell populations were decreased in the HNSCC tumors following BME feeding (7), in agreement with our current data. In addition, elevated expression of PDCD1/PD-1 in cancer samples indicated modulation of immune checkpoint regulation during carcinogenesis. Upregulation of the PD-1 receptor was reported in various human cancers, including oral cancer (16). Targeting PDCD1/PD-1 or its signaling pathway showed effective chemotherapeutic effect in phase I–III clinical trials against HNSCC (11). Thus, significant reduction of the proinflammatory and immune checkpoint molecules using bitter melon along with current therapy may have significant preventive effect for recurrence or migration of distant tumor (metastasis).

Recently, Tang and colleagues (32) reported that pathways of “cell-cycle progression” and “disruption of ECM and basement membrane” are activated at an early stage of tongue carcinogenesis. We also observed higher expression of MMP9 in 4-NQO–treated group. Interestingly, a significant lower expression of MMP9 was noted when compared with the BME-fed group. MMP9 is involved in different biological processes, such as ossification, invasion, and metastasis, and its elevated expression was reported in human oral cancer (17, 18). Therefore, BME treatment may play a major role in limiting these processes.

Furthermore, cancer cells frequently show alteration in cellular metabolism (33). Our RNA-Seq data suggested that BME feeding modulates lipid metabolism–associated gene expression as compared with 4-NQO–induced OSSC. Different in vitro and in vivo studies also reported a potential beneficial effect of bitter melon against lipid and glucose metabolic dysfunction (34). Thus, regulation of metabolism is an important mechanism of BME-mediated oral cancer chemoprevention and requires further investigation.

In summary, our study strongly demonstrated that oral feeding of BME prevents the incidence of carcinogen-induced tongue cancer by modulating different biological processes, including those of the immune system. This is the first study clearly indicating transcriptome modulation of different biological processes during BME-mediated chemoprevention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Sur, M. Varvares, R.B. Ray
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