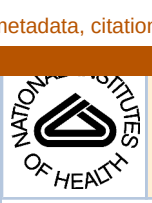

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Topical Application of a Bioadhesive Black Raspberry Gel Modulates Gene Expression and Reduces Cyclooxygenase 2 Protein in Human Premalignant Oral Lesions

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Abstract

Reduced expression of proapoptotic and terminal differentiation genes in conjunction with increased levels of the proinflammatory and angiogenesis-inducing enzymes, cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), correlate with malignant transformation of oral intraepithelial neoplasia (IEN). Accordingly, this study investigated the effects of a 10% (w/w) freeze-dried black raspberry gel on oral IEN histopathology, gene expression profiles, intraepithelial COX-2 and iNOS proteins, and microvascular densities. Our laboratories have shown that freeze-dried black raspberries possess antioxidant properties and also induce keratinocyte apoptosis and terminal differentiation. Oral IEN tissues were hemisected to provide samples for pretreatment diagnoses and establish baseline biochemical and molecular variables. Treatment of the remaining lesional tissue (0.5 g gel applied four times daily for 6 weeks) began 1 week after the initial biopsy. RNA was isolated from snap-frozen IEN lesions for microarray analyses, followed by quantitative reverse transcription-PCR validation. Additional epithelial gene-specific quantitative reverse transcription-PCR analyses facilitated the assessment of target tissue treatment effects. Surface epithelial COX-2 and iNOS protein levels and microvascular densities were determined by image analysis quantified

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Disclosure of Potential Conflicts of Interest

Dr. Russell Mumper is a co-Founder and Director of NanoMed Pharmaceuticals, and Dr. Susan Mallery is a member of NanoMed's Scientific Advisory Board. At the present time, NanoMed is negotiating with Ohio State University and The University of Kentucky to obtain an exclusive license agreement to the gel technology described in this report. NanoMed also has an awarded STTR grant in which Dr. Mallery is the PI. The subject of the awarded STTR is related to the topic of the gel technology; however, no data of any kind funded via the STTR is contained in the current article. Importantly, all of the data and studies described in the current report were planned, completed, and funded independently of NanoMed. The other authors disclosed no potential conflicts of interest.

immunohistochemistry. Topical berry gel application uniformly suppressed genes associated with RNA processing, growth factor recycling, and inhibition of apoptosis. Although the majority of participants showed posttreatment decreases in epithelial iNOS and COX-2 proteins, only COX-2 reductions were statistically significant. These data show that berry gel application modulated oral IEN gene expression profiles, ultimately reducing epithelial COX-2 protein. In a patient subset, berry gel application also reduced vascular densities in the superficial connective tissues and induced genes associated with keratinocyte terminal differentiation.

Introduction

Oral squamous cell carcinoma (SCC) is a worldwide health problem that is associated with significant morbidity, mortality, and an economic cost that rivals or exceeds other solid tumors (1–3). Recognized risk factors for the development of oral SCC include tobacco and alcohol use, poor oral hygiene, and potentially high-risk human papilloma virus subtypes (4,5). Other data also imply a role for sustained inflammation in oral SCC pathogenesis (6,7). Increased expression of two inflammation-induced proinflammatory enzymes, cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), is associated with malignant transformation of precancerous oral lesions (8–10). Notably, high levels of COX-2– and iNOS-generated reactive oxygen and nitrogen species can promote tumorigenesis by a variety of mechanisms (6,7). Oxidative and nitrosative stresses can induce nuclear and mitochondrial DNA mutations and inactivation of key cytoprotective enzymes such as manganese superoxide dismutase (11). In addition, increased COX-2 activity is associated with the suppression of apoptosis, oxygenase-mediated bioactivation of carcinogens, and reductase-induced conversion of compounds to potential mutagens (12). iNOS activation induces angiogenesis via nitric oxide's ability to serve as a signaling molecule for vascular endothelial growth factors (VEGF; ref. 13), and up-regulates basic fibroblast growth factor (14). Finally, as nitric oxide directly activates both of the COX isoforms (15), there is a positive correlation between cellular nitric oxide levels and COX function.

Oral intraepithelial neoplasia (IEN), histopathologically described as grades of epithelial dysplasia, is the recognized precursor to oral SCC. Oral IEN lesions present as white, red, or erythroleukoplakic patches (16). Although up to 36% of oral IEN lesions undergo malignant transformation, we cannot predict which lesions will progress (17). Although complete surgical excision remains the standard of care for advanced lesions, i.e., moderate dysplasia or higher, recurrences of lesional tissue or development of new premalignant lesions are not uncommon (18). Repeated surgeries increase costs and morbidity and negatively affect the ability to conduct clinical exams due to the accumulation of scar tissue.

Chemoprevention is a potential primary or adjunctive oral IEN treatment strategy. Black raspberries are one food that has shown significant chemopreventive efficacy (19–29). Furthermore, the freeze-drying process concentrates black raspberries' bioactive constituents ~10-fold on a weight basis relative to the natural fruit (27). Freeze-dried black raspberries (FBR) contain appreciable quantities of many putative chemopreventive compounds including vitamins A, C, and E, folic acid, calcium, selenium, α and β carotene, ellagic acid, ferulic acid, coumaric acid, and quercitin in addition to multiple anthocyanins and phytosterols (20,24). Our data show the ability of FBR to suppress redox-mediated intracellular signaling (21), inhibit survival pathways in transformed cells (19), reduce production of proangiogenic cytokines (22,25), and stimulate apoptotic and terminal differentiation pathways (22). *In vivo*, dietary administered FBR suppressed carcinogenesis in both hamster cheek pouch (23) and rat esophageal cancer models (24–26), inhibited the expression of COX-2 and iNOS (26), and suppressed tumor-associated angiogenesis (25). In addition, phase I human clinical trials have shown that orally administered FBR were well-tolerated (27,28) and also showed

the potential for FBR metabolism following oral administration by the detection of berry metabolites in the urine (29). Recently, our laboratories formulated a 10% FBR (w/w) bioadhesive berry gel for application to the human oral mucosa (30). Results from this clinical trial show that berry gel topical application significantly reduces loss of heterozygosity (LOH) indices at chromosomal loci associated with tumor suppressor genes in human oral IEN lesions without induction of any deleterious side effects (31).

We hypothesized that due to the anti-inflammatory and antioxidant properties of FBRs and their ability to suppress redox-mediated cell signaling, FBR gel application would be clinically active in oral IEN lesions. Accordingly, this study evaluated the effects of topical application of a 10% (w/w) FBR bioadhesive gel on oral IEN variables that included: histopathologic diagnosis, gene expression profiles, lesional epithelial COX-2 and iNOS levels, and microvascular density (MVD) of the superficial connective tissue. Our data show that berry gel application uniformly suppressed genes associated with RNA processing, growth factor recycling, and inhibition of apoptosis, and significantly reduced epithelial COX-2 levels. Furthermore, in a subset of patients, gel application also induced genes associated with apoptosis and keratinocyte terminal differentiation and reduced MVD.

Materials and Methods

Berry gel manufacturing

The bioadhesive gels used in this clinical trial were prepared using current good manufacturing practices at the cGMP facility within the Center for Pharmaceutical Science and Technology at the University of Kentucky, Lexington, KY. A description of the quality control measures and details of the gel composition and method of manufacture have been described previously (30). Briefly, the gel composition used for the clinical trial consisted of (in %, w/w): Noveon AA1 (NF; Noveon, Inc.) 1.35%, Carbopol 971P (NF; BF Goodrich Specialty Chemicals) 1.575%, glycerin (USP) 1.0%, edentate disodium (USP) 0.1%, 2-phenoxyethanol (BP) 1.0%, benzyl alcohol (USP) 1.0%, FBR 10%, and purified water as needed to obtain 100% (30). Based on our previous stability and mucosal penetration data (30), slight modifications of the prototype gel were made for the clinical trial gel. The FBR concentration was increased to 10% (w/w) and the pH of the gel was fixed at pH 3.5 to stabilize the more biologically active flavylium cation of the anthocyanin molecules.

Human clinical trials

Thirty-two adults (age range, 18–76 years) consented to participate in our clinical trial, which received approval from The Ohio State University Institutional Review Board (protocol 2003C0050). Criteria for inclusion as IEN participants were microscopically confirmed premalignant oral epithelial changes (noninvasive disease) and no use of tobacco products for 6 weeks prior to, or for the duration of, the clinical trial. Exclusion criteria included use of tobacco products within 6 weeks prior to or during the clinical trial, or a microscopic diagnosis of invasive oral SCC. Twenty participants had oral lesions that were clinically consistent with, and microscopically confirmed, as premalignant oral lesions (histopathologic diagnoses ranged from epithelial atypia to severe dysplasia, see Table 1A). The site, size, and consistency of premalignant lesions were recorded and clinical photographs obtained. Patient no. 4 (normal histology) and no. 16 (biopsy-confirmed invasive oral SCC) did not meet the study criteria and were therefore excluded from participation.

The remaining 10 individuals (five men and five women, aged between 24 and 34 years) exhibited clinically normal oral mucosa, and were included as trial participants (*a*) to allow additional observations for treatment-associated side effects, and (*b*) to evaluate the clinical and histopathologic effects of berry gel application on normal oral mucosa. The normal

participants' treatment protocol was identical to the IEN patients except that only a posttreatment biopsy was taken to reduce morbidity. Seven of these 10 normal participants had never used tobacco products or alcohol. The remaining three normal participants reported negligible (one participant) and modest alcohol (two participants) consumption with no tobacco use.

At the initial treatment appointment, half of the IEN tissue was excised, a portion placed in 10% neutral buffered formalin for histopathology and immunohistochemical studies, and a portion immediately frozen for microarray and reverse transcription-PCR (RT-PCR) analyses. This protocol ensured that pretreatment indices for each variable to be evaluated were obtained, thereby allowing each IEN participant to serve as their own internal control. Treatment entailed multiple dosing throughout the day, 0.5 g applied four times daily for 6 weeks for a total 84 g applied over the study duration. As human oral epithelium regenerates approximately every 28 days, a 6-week duration ensured the presence of FBR for at least one complete cycle of epithelial regeneration. Gel effects on LOH indices at loci associated with tumor suppressor genes in this cohort are reported separately (31).

Because the ventral-lateral tongue is a high incidence site for oral IEN and SCCs (4,6), this location was selected as the gel application site for the normal participants. All participants were monitored during the trial to observe for any adverse side effects, and in persons with premalignant lesions, any clinical progression in lesional tissue. All participants returned their used gel tubes at each weekly follow-up visit. Toxicity was assessed using National Cancer Institute common toxicity criteria version 3.0. Any toxicity equivalent to grade 2 or higher was considered dose-limiting.

Clinical photographs were taken, and biopsies obtained from the treated ventral-lateral tongue of the normal participants upon study conclusion. In the persons with premalignant lesions, excisional biopsies, which included the residual treated lesional tissue and initial biopsy site, were conducted. Tissue samples were handled as previously described.

Light microscopic diagnoses of all tissues were based on a seven-grade scale (normal, hyperkeratosis, atypia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma *in situ*, invasive SCC). In this study, the diagnosis "hyperkeratosis" alone conveyed a benign, reactive change without evidence of premalignant potential. In contrast, "atypia" signified architectural and cytologic alterations that in the clinical setting of an adherent demarcated white plaque represents early premalignant change. Two board-certified oral and maxillofacial pathologists reached agreement before a final histopathologic diagnosis was rendered.

RNA isolation and microarray analyses

Total RNA was isolated from snap-frozen patient biopsies using Absolutely RNA Miniprep Kit (Stratagene). The RNA concentrations were measured using Nanodrop ND-1000 (Nanodrop). The integrity of the total RNA was evaluated using capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies) and quantified using a Nanodrop 1000 (Nanodrop). Following confirmation of RNA quality, an Ovation Biotin RNA Amplification and Labeling System (NuGen Technologies, Inc.) was used to prepare amplified, biotin-labeled cDNA from total RNA following the instructions of the manufacturer. Briefly, first-strand cDNA was synthesized from 25 ng of total RNA using a unique first-strand DNA/RNA chimeric primer and reverse transcriptase. Following double-strand cDNA generation, amplification of cDNA was achieved by using an isothermal DNA amplification process that involves repeated SPIA DNA/RNA primer binding, DNA duplication, strand displacement, and RNA cleavage. The amplified SPIA cDNA was purified and subjected to a two-step fragmentation and labeling process. The fragmented/biotinylated cDNA content was measured in a ND-1000

spectrophotometer and the quality was analyzed on an RNA 6000 Nano LabChip (Agilent) using an Agilent Bioanalyzer 2100.

For each array, 2.2 μg of cDNA was hybridized onto Human Genome 133A GeneChips (Affymetrix, Inc.), containing probe sets that measure 22,000 transcripts from Human RNA. Sequences used in the design of the array were selected from GenBank, dbEST, and RefSeq. The sequence clusters were created from the UniGene database (Build 133, April 20, 2001) and were then refined by analysis and comparison with a number of other publicly available databases including the Washington University EST trace repository and the University of California, Santa Cruz Golden-Path human genome database (April 2001 release). Hybridization was conducted for 16 h at 45°C followed by washing and staining of microarrays in a Fluidics Station 450 (Affymetrix). GeneChips were scanned in a GeneChip Scanner 3000 (Affymetrix) and CEL files generated from DAT files using GeneChip Operating Software (Affymetrix). The probe set signals, which were generated using the RMA algorithm in ArrayAssist 3.4 (Stratagene), were used to determine differential gene expression by pair-wise comparisons. The genes that were altered by 1.5-fold either way were sorted and used for further interpretation of the microarray data. Microarray data were validated by conduction of quantitative RT-PCR analyses on three consistently down-regulated genes (*SFRS7, SFRS11,* and *LGALS8*) and five up-regulated genes (*KRT2B, DSC1, UGT2B, SPPR3,* and *UBD*), using matched pretreatment and posttreatment RNA from four donors for each gene evaluated. In order to establish which pathways were affected by gel treatment, microarray analyses were conducted midway through the trial on 13 matched pretreated and posttreated IEN samples.

Quantitative RT-PCR analyses

Total RNA (100 ng) was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). All gene-specific TaqMan MGB primer and probes (FAM dye-labeled) and human β-glucuronidase (β-GUS) endogenous control (VIC dye-labeled) were purchased from Applied Biosystems. Real-time PCR was performed using Applied Biosystems 7500 real-time PCR system. One microliter of cDNA was used in a total volume of 20 μL real-time PCR reaction containing 10 μL of TaqMan Universal PCR Master Mix, 1 μL of gene-specific TaqMan MGB primer and probe, and 1 μL of human β-GUS. The PCR was run with an initial AmpErase enzyme activation step at 50°C for 2 min, followed by a run at 95°C for 10 min and 40 cycles at 95°C for 15 s, and at 60°C for 1 min. The comparative C_T method was used to calculate RNA expression level. The amount of target, normalized to a β-GUS endogenous control and relative to the normal patients, is given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ (target) − ΔC_T (mean value of normal patients), ΔC_T is the C_T target gene subtracted from the C_T of β-GUS, and C_T is the threshold cycle.

Immunohistochemistry and image analysis for epithelial COX-2 and iNOS

COX-2 and iNOS protein staining was conducted on serially sectioned paraffin-embedded pretreatment and posttreatment specimens from all of the IEN patients. Endogenous peroxidase activity was blocked by pretreatment with 3% H_2O_2, followed by microwave processing to facilitate antigen retrieval. Sections were then treated with 5% normal serum, 1% bovine serum albumin, 0.05% Tween 20 for 1 h, and then incubated with either the primary antibody [goat anti–COX-2 polyclonal antibody, 2 μg/mL dilution (Cayman Chemical), or anti-iNOS rabbit polyclonal antibody, 1:500 dilution (Biomol International)] or PBS (negative control) at 4°C overnight. Vectastain ABC reagent (Vector Laboratories) was then applied and sections incubated for 30 min. Immunoreactions of methyl green counterstained sections were visualized using the VIP substrate (Vector). Images were captured using a DS-Fi1 5-megapixel color digital camera (Nikon). The percentages of positive staining areas were analyzed using Image-Pro Plus 6.2 software (Media Cybernetics). The area of interest was defined as the entire epithelium excluding the acellular keratin layer.

Immunohistochemical staining for CD34 and determination of MVD

From the 20 IEN biopsy samples, 11 matched sets retained sufficient tissue for further studies. These samples were stained and analyzed to determine MVD. Five-micron sections were mounted on SuperFrost Plus slides (Fisher Scientific) and then successively deparaffinized, rehydrated, treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase, and microwaved in citrated buffer (pH 6.0) for antigen retrieval. Sections were then blocked in 5% normal horse serum, 1% bovine serum albumin, 0.05% Tween 20 in PBS, incubated with anti-CD34 mouse monoclonal antibody (1:20, BioGenex), washed, and then incubated with biotinylated horse anti-mouse IgG (1:200) followed by horseradish peroxidase–conjugated avidin complex (Vectastain ABC kit, Vector Laboratories). The sections were then developed with 3,3′-diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin.

The CD34-stained tissue sections were captured at 200-fold image scale [microscopic field, 1,280 × 960 pixels (width × height), 300 dpi resolution] using a Nikon DS-Fi-1 5-megapixel digital camera and Image-Pro Plus software. The images were imported into Adobe PhotoDeluxe software, and displayed on a high-resolution 17-inch color monitor. The area of interest was defined as the connective tissue located 90 μm underlying the most inferior epithelial rete ridge. Microvessels were then manually counted on the computer screen. Any single brown-stained cell (CD34 positive) or cluster of endothelial cells was counted as a single vessel. MVD was calculated as the mean number of microvessels per area of interest in the microscopic computerized field (×200 magnification).

Statistical analyses

The effects of berry gel application on surface epithelial COX-2 and iNOS proteins were analyzed using a Wilcoxon sign rank test. Findings with $P < 0.05$ values were considered to be significant.

Results

Berry gel use elicited no deleterious effects

Toxic responses such as hematologic disturbances would not be anticipated with the use of a natural product. There were concerns, however, regarding potential complications such as a contact mucositis, or due to the sugar content in berries, Candidal infections. None of the 30 trial participants developed any adverse effects. Furthermore, the treated tissue sites in the normal tissue donors retained normal clinical and microscopic appearances (data not shown). As determined by the minimal residual gel in the returned berry gel tubes (>95% dose used), patient compliance was high.

Oral IEN lesion participant demographics and histopathologic diagnoses

Table 1A depicts the IEN patient demographics and histopathologic diagnoses. Follow-up information on a subset of these patients (Table 1B) following the clinical trial is discussed later. IEN patients were older than the normal tissue donors, and the majority had a previous history of tobacco and alcohol use. Seventy percent of the patients had either multifocal lesions and/or a history of recurrent disease at the selected treatment site, factors associated with a higher-risk cohort which is more recalcitrant to treatment. Histopathologic improvement was seen in seven patients' lesions, disease progression (increase) in grade in four patients, and nine patients' lesions exhibited no change (stable disease) in microscopic appearance. Lesional grade decreases were not restricted to lower grade lesions as the pretreatment diagnoses were severe or moderate dysplasia in 20% (4 out of 20) of the seven lesions that showed histologic improvement (Table 1A). The youngest IEN patient (no. 9), who had three previous diagnoses

of epithelial dysplasia at the treatment site (these biopsies preceded her entrance into the clinical trial), had complete clinical and microscopic lesional regression following treatment. This site has retained a normal histology 15 months following trial cessation (Table 1B). The posttreatment biopsy of patient no. 8 showed an increase of three grades, from mild dysplasia (pretreatment) to focal carcinoma *in situ* (posttreatment). Patient no. 8's lesional tissue was remarkable for its extremely subtle clinical appearance, which introduced the prospect of sampling error at the pretreatment biopsy.

Clinical measurements were not used as therapeutic indicators due to our study design (lesional tissues hemisected to provide baseline variables) and the fact that the clinical appearances of oral IEN lesions are dynamic and can vary from week to week, regardless of treatment (17). Although pretreatment IEN lesions ranged from 0.3 × 0.3 to 2.5 × 1.5 cm (Table 1A), pretreatment sizes had no apparent effects on therapeutic responsiveness.

Modulation of oral IEN gene expression profiles by berry gel application

Functional annotation groupings and their corresponding Benjamini scores were used to identify genes consistently down-regulated following gel treatment. Twenty-seven genes, which encompassed RNA processing, signal transduction, and inflammatory pathways showed uniform down-regulation (mean decrease ≥ 1.5-fold) following berry gel application (Table 2). The majority (13) of the down-regulated genes involved RNA processing and included transcriptional activation, RNA splicing, and nuclear export. Other down-regulated genes included DUSP5, CTSL2, PIGL (signaling pathways) and LGALS8, RNASET2, and CFLAR, which function in integrin-associated interactions, chromosomal rearrangements, and inhibition of apoptosis, respectively. These latter three genes are frequently up-regulated in cancers (32,33).[8] The remaining down-regulated genes serve in protein trafficking, and proinflammatory responses. The RT-PCR validation studies showed comparable fold changes to those detected by microarray analyses (Table 2).

Up-regulation of apoptotic and terminal differentiation genes occurred in a subset of patients

Although consistent down-regulatory effects were identified, posttreatment increases in gene expression showed greater variability. Microarray analyses revealed that a subset of patients' IEN lesions responded to berry gel application with up-regulation of proapoptotic and terminal differentiation pathways (Table 3). The gene panel selected for RT-PCR analyses therefore included these microarray-identified genes in addition to genes that had either protumorigenic functions, e.g., *COX-2, iNOS, VEGF*, or were associated with epithelial terminal differentiation. As is apparent from the data shown in Table 3, there was considerable interpatient heterogeneity with regard to which genes showed a modulation in expression and the magnitude of the effect(s). Consistent with the findings of other investigators (34,35), data from Table 3 reveals that genes which showed an appreciable increase by microarray analyses showed an even greater expression increase when evaluated by RT-PCR.

Topical black raspberry gel reduces the levels of COX-2 and iNOS proteins in treated lesional epithelium

COX-2 and iNOS proteins showed distinct and different intraepithelial distributions. Furthermore, the relative intensities for both COX-2 and iNOS varied from patient to patient and treatment status. COX-2 distribution showed an epithelial layer specificity, i.e., COX-2 was located in the spinous and granular layers, with negligible COX-2 immunoreactivity observed in the basal cells (Fig. 1). In contrast, iNOS was more diffusely distributed throughout

[8] http://www.ncbi.nlm.nih.gov/sites/entrezgene

the surface epithelium and some tissues showed iNOS positivity within the basal layer cells (Fig. 1).

Seventeen of the 20 matched pretreatment and posttreatment samples showed a significant posttreatment decrease in COX-2 ($P < 0.005$; Table 4). Although a majority (12 of 20) of participants showed posttreatment decreases in epithelial iNOS, these differences were not statistically significant ($P = 0.53$; Table 4). Finally, the extent of gel application effects on epithelial COX-2 and iNOS levels varied appreciably among patients (Table 4).

Superficial connective tissue MVD is diminished in some treated oral IEN lesions

Based on sample availability, MVD analyses were conducted on 11 pretreatment and posttreatment matched tissues. Seven participants showed a decrease in MVD following gel treatment (extent of decreases ranging from −2.9% to −67.3%; Table 4). Four of these positive responders, which exhibited higher percentage MVD decreases [patient nos. 2 (−67.3%), 7 (−38.9%), 15 (−28.9%), and 20 (−19.8%)], also showed decreases in *VEGF* expression (−10, −5.4, −1.1, and −1.8, for patient nos. 2, 7, 15 and 20, respectively; Tables 3 and 4; Fig. 2A and B). Notably, the *VEGF* RT-PCR data from the three low positive responders (nos. 3, 10, and 14) and the four patients (nos. 13, 18, 19, and 21) which showed MVD increases (increases ranging from +7.7% to +44.1%), also showed decreased *VEGF* expression in five patients, three of which showed decreases of 2-fold or greater. These *VEGF* data likely reflect the contribution of numerous factors in addition to transcriptional regulation that affects vascular density.

Summarized data of black raspberry clinical trial reveals a cohort of “high” responders

The data presented in Table 4 depicts the overall effects of berry gel application on the 20 patients with oral IEN. The corresponding LOH indices data, which are discussed in a separate report, are included for completeness (31). Three of the 20 patients (nos. 2, 9, and 12) showed a high overall therapeutic responsiveness as determined by a >45% positive response rate. Common features of the “high” responders were decreased iNOS protein (−27.7%, −24.1%, and −16.0%), high responsiveness with respect to gene modulation (9, 6, and 8, respectively), and no increases in histopathology (two decreased grade and one stable disease). The one high-response participant (no. 2) who had sufficient tissues for MVD analyses also showed the greatest posttreatment reduction (67.3%) in vascularity. The uniform features of the “low” responders (≤30% positive response rate, nos. 5, 6, 8, 11, 15, 17, 18, and 21) were a posttreatment increase in iNOS proteins in five of eight patients, either no change (nos. 6, 15, 18, 21) or an increase (nos. 8, 11, 17) in posttreatment histologic grade in seven of eight patients, and modest to low gene-modulatory effects in eight of eight patients. Collectively, 60% of our participants showed >30% positive therapeutic responses following treatment.

Follow-up information on clinical trial participants

Follow-up information regarding study participants (Table 1B) is limited by the complexion of the patient pool in this regional referral center. Eleven patients remained as patients of record at Ohio State and follow-up on these individuals ranged from 3 to 23 months. Three participants (nos. 5, 9, and 15) have had no recurrences at the treated site with disease noted at other sites. Three patients (nos. 3, 7, and 19) exhibited no change in disease status at the treatment site with two of these three patients (nos. 3 and 19) developing lesions at other locations. Four participants (nos. 1, 2, 10, and 14) showed disease progression at the treatment site, one patient (no. 1) developed invasive SCC, and two patients (nos. 2 and 14) developed involvement of other sites. The follow-up 7-month posttreatment biopsy of participant no. 21 showed a histologic grade decrease at the treatment site.

Discussion

Our results show that application of a bioadhesive 10% FBR gel to oral IEN lesions showed therapeutic efficacy without the induction of any adverse effects. These data further validate the established safety record for the two cross-linked polyacrylic acid–based polymers used as either the bioadhesive or the gel base (30) and are consistent with previous human studies conducted by our laboratories, which used dietary berry administration (27–29).

Histopathology data show variability in oral IEN responsiveness, with 35% of our participants demonstrating a lesional grade decrease, 20% of participants showing a lesional grade increase, and 45% of the patients' lesions exhibiting stable disease. Although modest, these histologic response rates are comparable with previous oral IEN chemoprevention trials (36,37). A previous study by Papadimitrakopoulou and colleagues, which evaluated the effects of 13-cis retinoic acid, α-tocopherol, and IFN-α on oral and laryngeal IEN lesions, revealed that only 14% (one of seven) of the oral dysplastic lesions showed partial histologic regression at the completion of the 12-month study, with 45% (5 of 11) demonstrating either partial or complete regression at the intermediate 6th month assessment (36). As the rates of histologic regression were higher in the laryngeal lesions, the investigators speculated that the more recalcitrant oral cavity lesions had sustained different, potentially greater, molecular perturbations (36). Similarly, another clinical trial which evaluated an ONXY-15–containing mouthwash that targeted p53-defective epithelial cells reported a 37% histologic regression rate (37).

Microarray analyses revealed a consistent down-regulation in genes associated with RNA processing, intracellular signaling, and antiapoptotic and proproliferative pathways. As indicated by the number of genes affected, the greatest reduction occurred in RNA processing genes, which could ultimately affect both transcription and translation (38). Notably, several putative tumor-promoting genes showed posttreatment down-regulation including heterogeneous ribonucleotide protein A1 (hnRNPA1). *hnRNPA1* functions in diverse biochemical interactions that include an overall regulation of transcription, augmentation of telomerase activity, and increased IκBα degradation resulting in nuclear factor κB (NFκB) activation (38). The other tumor-associated genes showed reduced expression function in integrin-associated cell interactions (*LGALS8*), facilitation of chromosomal rearrangement (*RNASET2*), and inhibition of apoptosis (*CFLAR*). These microarray data suggest that FBR treatment diminishes overall transcriptional activity, resulting in less biochemically active cells. Indeed, modulation towards more differentiated, less proliferative gene expression profiles were observed in a subset of patients following gel application. As previous oral cavity microarray studies have focused on obtaining "signature" gene expression profiles of normal, premalignant and malignant oral tissues in the absence of treatment, direct comparisons with our study are not feasible (34,35,39). In a recent esophageal chemoprevention trial, Joshi and colleagues evaluated pretreatment and posttreatment gene expression profiles in histologically normal esophageal tissues in 29 patients with premalignant esophageal lesions and identified modified expression of 15 genes (40). Ten of these genes, which were associated with inflammation and immune suppression, showed up-regulation during lesional progression, whereas the expression of five genes associated with immune stimulation increased with regression (40).

Our RT-PCR analyses revealed a large interpatient response range. Provided the outbred human population, heterogeneity among premalignant lesions, and complexity of gene regulation, these data were not surprising. Previous studies from our laboratories provide mechanistic insights as to how FBR could modulate oral IEN gene expression and induce the observed downstream effects (19–26). Huang and colleagues showed that a FBR methanol extract reduced activator protein 1 (AP-1) activation by targeting mitogen-associated protein kinase pathways including ERKs, JNKs, and p38K, and also suppressed NFκB activation by

inhibiting IκBα phosphorylation (21). Notably, three potentially protumorigenic genes, i.e., *COX-2*, *iNOS*, and *VEGF*, all have NFκB-binding sites (40). Furthermore, AP-1 activation is associated with up-regulation of *COX-2* and *VEGF* (41). Our observed posttreatment decreases in COX-2 and iNOS proteins, and the reduction of vascular densities, are therefore consistent with FBR's suppression of AP-1 and NFκB activation. The methanol FBR extract also inhibited the activation of a key cell survival and proangiogenic signaling pathway, i.e., phosphoinositide-3-kinase/Akt (19). Additional studies, which showed that an ethanol FBR extract reduced VEGF production and increased the functional activities of caspase 3 and keratinocyte-specific transglutaminase in human oral SCC cells, showed FBR's downstream effects on apoptotic, differentiation, and angiogenic proteins (22).

The majority of IEN patients showed decreases in epithelial COX-2 and iNOS proteins following treatment. These data are consistent with our previous results which showed that dietary FBR administration reduced COX-2 and iNOS proteins in a rat esophageal cancer model (26). Questions arise, however, regarding what extent of decreases are pathophysiologically significant. Perhaps equally or potentially more important, are the COX-2 and iNOS enzyme levels relative to the reactive species degrading enzymes within critical cell populations. High COX-2 and iNOS activities in rapidly dividing cells, such as the transient amplifying cell population, or in the stem cell pool, have the potential to induce consequential, tumor-promoting nuclear and mitochondrial mutations (7,12). Furthermore, high production of prostanoids and reactive nitrogen species by cells in communication with the underlying connective tissue facilitates angiogenesis (7,12). Our MVD results did not reveal any clear associations between the treatment effects on VEGF expression and the corresponding MVD. These data likely reflect the varied factors associated with angiogenesis, including multiple VEGF splice variants which may have varying biological efficacies, translational and posttranslational modifications of proangiogenic proteins, local levels of proangiogenic factors, and the requirement for activated endothelial cells (42).

The extent of therapeutic efficacy necessary to be deemed a positive outcome in human clinical trials remains to be defined. A recent oral IEN clinical trial stipulated a priori that their tested agent would be considered active if 14.3% of their patients showed a positive response (43). Our clinical trial results identify a subset of high therapeutic responders, and also indicated that 60% of our patients showed a >30% positive response rate. Several factors such as sustainability and penetration of compounds at the treatment site and compound stabilization or metabolic bioactivation at the target tissues all likely affect clinical outcomes. It is also feasible that an optimized dose or dosing schedule may improve our therapeutic responses. Our follow-up data, which implies that longer duration treatment will likely be necessary for patients with oral IEN, emphasizes the need to identify both effective and well-tolerated agents.

Previous oral IEN clinical trials have relied primarily on systemic agent administration, resulting in some toxicities and concerns regarding sufficient agent availability at the treatment site (36,43–45). Although local delivery via a mouthwash was recently used in an oral IEN trial, this study was discontinued after a patient developed circulating antibodies to the adenovirus vector (37). Aspects of this current study, i.e., the local delivery of naturally derived chemopreventives, have modified the oral IEN chemoprevention paradigm. Finally, although this trial should be considered exploratory due to its modest size and relatively short duration, therapeutically promising data have been obtained.

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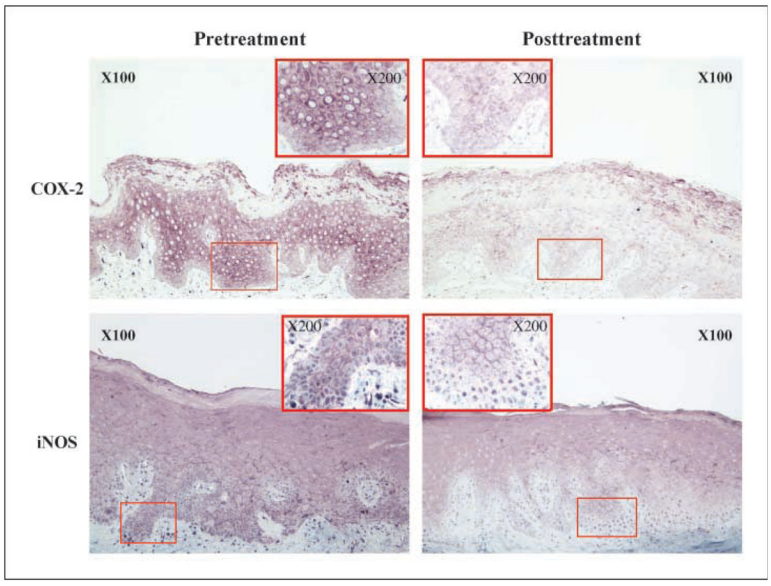

Figure 1.

Although the majority of patients with oral IEN showed a reduction in epithelial levels of COX-2 and iNOS at the conclusion of the trial, the extent of protein decreases varied among patients. The pretreatment COX-2 photomicrograph of patient no. 20 shows moderate to intense positivity in the spinous and granular layers, which was markedly reduced following treatment (88.9% decrease for patient no. 20). In contrast, iNOS showed a more uniform distribution throughout the epithelium, inclusive of basal cell staining, as is apparent in the pretreatment iNOS photomicrograph for patient no. 9. This patient showed an overall high therapeutic response rate (47.4%), inclusive of a 24.1% decrease in epithelial iNOS following berry gel application (all photomicrographs taken at × 100 image scale, using an Olympus BX51 microscope equipped with a Nikon DS-Fi1 digital camera).

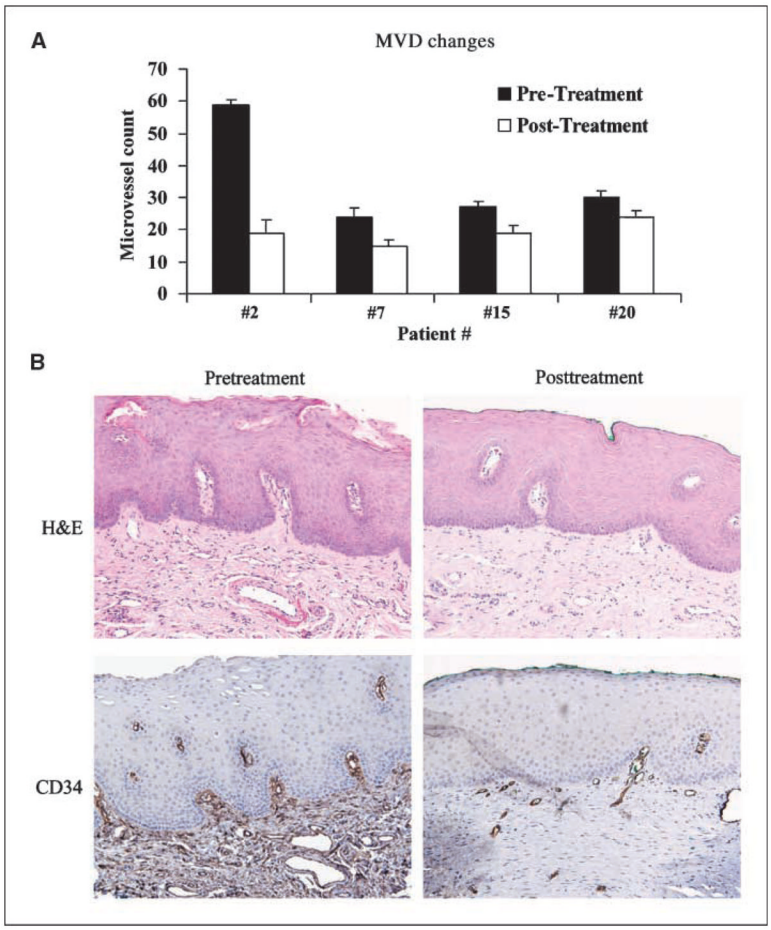

Figure 2.

Eleven clinical trial participants retained sufficient pretreatment and posttreatment tissues for the evaluation of the effects of berry gel on MVD. *A*, seven of the 11 participants showed posttreatment decreases, with four participants showing MVD reductions that ranged from 19.8% to 67.3%. *B*, pretreatment and posttreatment photomicrographs of patient no. 2. This individual showed the highest overall therapeutic response rate of 60%, including a decrease in lesional histologic grade apparent in the H&E-stained sections (pretreatment diagnosis—mild dysplasia; posttreatment diagnosis—atypia) and a 67.3% reduction in MVD, as noted in the pretreatment and posttreatment CD34 sections (all photomicrographs taken at ×100 image scale using an Olympus BX51 microscope equipped with a Nikon DS-Fi1 digital camera).

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Table 1

Patient demographics

(A) Oral IEN patient demographics and histopathologic diagnoses

Patient no.	Age (y)	Patient and lesion characteristics				Multifocal[§]	Recurrent[‖]	Size (cm)[¶]	Histopathologic diagnosis	
		Gender	Smoking[*]	Alcohol[†]	High-risk site[‡]				Pretreatment diagnosis	Posttreatment diagnosis
1	74	M	Y	N	N	N	N	2.3 × 0.8	Severe dysplasia, can't rule out superficially invasive SCC	Primarily moderate, focally severe
2	75	M	Y	Y	Y	Y	Y	1.8 × 0.8	Mild dysplasia	Hyperparakeratosis, atypia
3	59	M	Y	Y	N	Y	Y	1.0 × 1.0	Severe dysplasia	Moderate dysplasia
5	75	F	Y	N	N	Y	N	0.8 × 0.6	Mild dysplasia	Hyperkeratosis, atypia
6	44	M	N	Y	N	N	Y	0.6 × 0.4	Hyperkeratosis, atypia	Hyperorthokeratosis, atypia
7	58	M	N	Y	Y	N	Y	2.1 × 0.6	Moderate dysplasia	Moderate dysplasia
8	76	F	Y	Y	Y	N	N	1.2 × 0.5	Mild dysplasia	Focal carcinoma *in situ*
9	26	F	Y[**]	Y	N	Y	Y	0.3 × 0.3	Hyperkeratosis, atypia	Hyperkeratosis
10	66	M	Y	N	Y	N	N	2.2 × 1.2	Moderate dysplasia	Hyperparakeratosis, atypia
11	59	F	Y	N	N	Y	N	2.0 × 1.0	Hyperkeratosis, atypia	Mild dysplasia
12	56	F	N	N	N	Y	N	1.2 × 1.0	Hyperkeratosis, atypia	Hyperkeratosis, atypia
13	59	F	N	Y	Y	N	Y	2.2 × 0.6	Hyperkeratosis, atypia	Hyperkeratosis, atypia
14	44	F	N	Y	Y	Y	Y	2.2 × 0.8	Severe dysplasia	Moderate dysplasia, candida
15	43	M	Y	N	N	Y	N	1.2 × 0.5	Hyperkeratosis, atypia,	Hyperkeratosis, atypia,
17	55	F	Y[c]	Y	N	Y	Y	0.8 × 0.4	Mild dysplasia	Moderate dysplasia
18	73	F	Y	N	Y	Y	N	2.0 × 1.0	Focal moderate dysplasia	Moderate dysplasia
19	64	F	Y	Y	Y	N	Y	2.5 × 1.5	Focal moderate dysplasia	Focal severe dysplasia
20	62	M	Y	Y	N	Y	N	2.0 × 1.0	Mild dysplasia	Mild dysplasia
21	58	F	Y	Y	N	N	N	2.5 × 1.0	Moderate dysplasia	Moderate dysplasia
22	53	F	Y	N	N	N	N	1.0 × 1.0	Hyperkeratosis, atypia	Hyperkeratosis, atypia
	Range	Totals	Totals	Totals	Totals	Totals	Totals		Histopathologic change	
	26–76 (mean, 59.0)	M = 8/F = 12	Y = 15/N = 5	Y = 12/N = 8	Y = 8/N = 12	Y = 11/N = 9	Y = 9/N = 11		Decrease in grade = 7 (35%), increase in grade = 4 (20%), stable disease = 9 (45%)	

(B) Clinical trial disease status follow-up information

Patient no.	Follow-up (mo)	Treatment site††	Other oral sites
1	7	Progression to well-differentiated SCC (moderate, focally severe dysplasia)	No other lesions observed
2	23	Mild dysplasia (atypia)	Mild dysplasia at one new site
3	13	Moderate dysplasia (moderate dysplasia)	Developed verrucous carcinoma at two sites
5	18	No recurrence‡‡	Atypia at one new site
7	20	Moderate dysplasia (moderate dysplasia)	No other lesions observed
9	15	No recurrence‡‡	Atypia at one new site
10	16	Mild dysplasia (atypia)	No other lesions observed
14	10	Severe dysplasia (moderate dysplasia posttreatment-former resection site of oral SCC)	Atypia at one new site
15	3	No recurrence‡‡	Atypia at several new sites
19	3	Moderate to severe dysplasia (focally severe dysplasia)	Moderate dysplasia at one new site
21	7	Atypia (moderate dysplasia)	No other oral lesions observed

*"Y", history of smoking; "N", no history of smoking.

†"Y", current alcohol use; "N", no alcohol use.

‡"Y", Lesion location at high risk for progression to SCC (lateroventral tongue, floor of mouth, tonsillar pillar); "N", lesion location at lower risk for progression to SCC (buccal mucosa, gingiva, hard/soft palate).

§"Y", multiple IEN lesions observed clinically; "N", isolated clinical disease.

||"Y", selected site represents recurrent disease; "N", selected site presents new disease.

¶Pretreatment lesional size.

**Indicates quit smoking ≤1 year ago.

††For recurrent lesions, the diagnosis at follow-up is listed first and then succeeded by posttreatment diagnosis in parentheses as previously listed in Table 1A.

‡‡No recurrent lesional tissue was identified clinically.

Table 2

Genes down-regulated as determined by microarray analyses

Gene and gene ID	Gene function	Patient no.													No. of patients ≥ 1.5
		1	2	3	5	6	7	8	9	10	11	12	13	14	
SFRS 7 6432	Shuttles serine/arginine-rich splicing factors, and an adaptor for mRNA nuclear export	−1.4	−2.1	−1.5	−2.1	−3.3 (−2.2)	−4.0	−1.5 (−1.1)	−1.6 (−1.3)	−1.4	−3.7	1.1	−2.5 (−1.2)	−2.1	10
SFRS 11 9295	Functions in pre-mRNA processing	−1.9	−2.3	−2.5	1.2	−2.0 (−1.8)	−2.1	−1.6 (−1.4)	−3.7 (−1.3)	−1.7	−4.6	−1.1	−2.5 (−1.2)	1.3	10
SFRS 5 6430	An adaptor for mRNA nuclear export, interacts with export receptor TAP	−1.1	−1.3	−1.2	−1.4	−1.5	−2.0	−1.4	−2.0	0.0	−1.3	0.0	−1.7	−1.7	5
hnRNPA1 3178	Functions in pre-mRNA processing and other aspects of mRNA metabolism and transport	−1.2	−1.9	−1.6	−1.5	−1.7	−1.6	1.2	−2.8	−1.9	−3.7	−2.5	−3.3	−1.2	10
IL-18 3606	Proinflammatory cytokine, can induce IFN-γ production	−2.0	−1.7	−1.9	−2.1	−3.5	−1.5	1.1	−4.9	−1.9	−1.7	−1.9	−1.3	1.5	10
ZNF83 55769	Serves in zinc binding, nucleic acid binding, and transcription factor activity	−2.1	−1.3	−2.1	−1.3	−1.7	−1.7	−1.2	−2.5	−3.5	−2.5	−1.3	−2.0	1.7	7
LGALS8 3964	This protein is widely expressed in tumor tissues, involved in integrin-associated cell interactions	−3.0	1.1	−1.2	−1.9	−3.3 (−2.3)	−2.5	−1.3 (−1.1)	−2.6 (−1.5)	−1.6	−1.9	−1.9	−1.6 (−1.4)	1.6	9
DUSP5 1847	Negative regulator of mitogen-associated protein kinase superfamily, dephosphorylates phosphorylated serine, threonine and tyrosine residues	−1.6	−1.6	−1.1	1.2	−2.3	−2.8	−2.9	−2.3	−1.2	−2.5	1.1	−1.3	−4.0	8
CLK1 1195	Functions in splicing site selection in the nucleus	−1.3	−2.1	−2.0	1.1	−2.1	−1.3	−1.5	−1.7	−1.2	−3.3	−1.4	−2.6	−1.4	7
MRS2L 57380	Mg^{2+} transporter which maintains mitochondrial Mg^{2+} concentrations	−1.9	−1.9	−1.3	−1.2	−2.5	−1.4	−2.3	−2.1	−1.5	−2.5	−1.9	−1.3	1.2	8
RNASET2 8635	A secreted RNase, associated with human cancers and chromosomal rearrangement	−2.1	−1.6	−2.3	−1.2	−1.7	1.5	−1.1	−2.3	−2.1	−2.3	−1.9	−2.0	−1.2	9
TBCE 6905	Protein used β-tubulin folding and microtubule formation, trafficking of golgi and endosomal proteins	−1.7	−2.5	−1.7	−1.2	−1.9	−1.6	−1.3	−2.0	0.0	−1.9	−2.6	−2.1	1.4	9

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Gene and gene ID	Gene function	Patient no.													No. of patients ≥ 1.5
		1	2	3	5	6	7	8	9	10	11	12	13	14	
SFPQ 6421	Polypyrimidine tract binding protein-splicing factor proline/glutamine-rich	−1.2	−1.6	−2.0	−1.6	−3.3	−1.4	0.0	−2.5	−1.1	−3.5	−1.3	−1.5	1.1	7
CCNL2 81669	Functions in transcription and RNA processing	−1.2	−1.2	1.1	−2.0	−2.5	−2.5	−1.6	−3.3	1.1	−3.0	−1.1	−1.4	−1.3	6
SLC25A28 81894	Mitochondrial protein involved in solute transport	−1.1	1.1	−1.2	−1.2	−2.6	−2.6	−1.7	−2.3	−1.7	−2.8	−1.2	−1.5	−1.2	7
CTSL2 1515	Lysosomal cysteine protease, putatively functions in growth factor recycling, increased expression in cancers	−1.9	−1.6	−1.4	−2.8	−2.8	−1.4	−1.9	−1.5	−1.4	−1.5	−1.2	1.7	−1.3	7
GAPDS 26330	Gyceraldehyde-3 phosphate dehydrogenase, generates 1,3 diphosphoglycerate	−1.9	−2.0	1.3	−1.9	−1.7	−2.8	0.0	−1.4	−1.5	−1.7	−1.3	−2.0	1.1	8
WIPI49 55062	Regulates the assembly of multiprotein complexes and interacts with phospholipids	−1.7	−3.3	−1.4	−1.2	−1.6	−1.3	−1.9	−1.3	−1.2	−2.3	1.2	−1.7	−1.2	6
PIGL 9487	Functions in phosphatidylinositol glycan synthesis	−1.4	−2.0	0.0	1.2	−1.9	−2.5	−1.9	−1.9	−1.2	−2.3	−1.2	−1.7	0.0	7
MRPL48 51642	Mitochondrial ribosomal protein, assists in mitochondrial protein synthesis	−1.7	−1.9	0.0	−1.3	−2.0	−1.3	−1.4	−1.5	−1.1	−2.6	−1.3	−1.4	−1.2	5
RBM34 23029	RNA binding motif protein 34	−1.9	−1.9	−1.4	−1.3	−1.9	−1.1	−1.3	−1.9	−1.4	−2.8	1.2	−1.6	1.2	6
MRPS31 10240	Mitochondrial ribosomal protein S31, functions in translation initiation	−1.2	−1.9	−1.1	1.1	−1.9	−1.2	−1.2	−2.1	−1.5	−1.6	−2.3	−1.6	0.0	7
CFLAR 8897	Serves as an inhibitor of apoptosis, speculated to function as a tumor progression factor	−1.2	−1.6	−1.5	−1.1	−1.3	−2.0	−1.7	−1.9	1.2	−1.4	−1.4	−1.3	−2.1	5
MED6 10001	Mediator of RNA polymerase I transcription	−1.4	−1.2	−1.2	−1.4	−2.0	−1.5	−1.5	−2.3	−1.2	−1.7	1.2	−1.2	−1.3	5
NACA 4666	Serves as a transcriptional coactivator and augments AP-1 activity	−1.2	−2.1	−2.0	−1.7	−2.3	−2.0	−1.3	−1.2	−1.5	−2.3	1.1	−2.3	1.4	8
KIAA0117 23029	Nucleolus associated RNA binding motif protein	−1.9	−1.9	−1.4	1.3	−1.9	−1.1	−1.3	−1.9	−1.4	−2.8	1.2	−1.6	1.2	6
PTS 5805	Functions in tetrahydrobioterin synthesis, serves as a cofactor for nitric oxide synthase isoforms	−1.1	−1.7	−1.5	−1.6	−2.1	−1.5	−1.2	−1.9	−1.1	−2.0	0.0	−1.5	1.2	8

NOTE: Data expressed as fold change in the posttreatment tissues relative to participant-matched pretreatment tissues. Values in parentheses are confirmatory data obtained by quantitative RT-PCR (patient nos. 6, 8, 9, and 13).

Table 3

Effects of topical berry gel application on epithelial-specific genes as assessed by quantitative RT-PCR

Gene and gene ID	Gene function	Patient no.																		
		1	2	3	5	6	7	8	9	10	11	12	13	14	15	17	18	19	20	21	22
KRT2B 51350	Major cytoskeletal protein of suprabasilar epithelium	−1.8	75.5 (27.9)	−12.6	13768 (78.8)	−1.7 (−1.9)	3	9	8.3	46.2	−2.5	6.4	1.8 (1.4)	6.8	78	32.8	3	−1.0	4.10	2.9	5.6
DSC1 1823	Epithelial cell adhesion & desmosome formation	−2.1	47.2 (21.1)	−17.9	−2.8 (−1.2)	2.2 (1.74)	6.8	2.8	−1.3	1.1	−1.8	2.7	2.70 (1.9)	150	6.3	−2.9	−1.2	−5.0	−1.1	5.3	1.500
UGT2B 54490	Phase II detoxifying enzyme, increases agent solubility	1.00	2.6 (3.7)	−1.1	1.2 (5.3)	12 (8)	1.3	1.80	4.60	1.4	3.10	8.9	1.6 (1.2)	2.3	−4.6	1	−1.6	−1.3	−1.1	−9.9	−50.2
KSR1 8844	Kinase suppressor of ras1	−1.8	2.6	1.000	1.1	−1.4	1.5	−1.7	−1.2	−1.6	1.8	1.5	1.5	2.6	−2.4	−4.5	1.3	2.5	2.0	−3.5	−1.3
PPP2CA 5515	Protein phosphatase, functions in negative control cell growth	1.7	−1.1	−1.1	−1.4	−1.3	−1.2	1.1	1.4	1	−1.3	0.8	−1.5	1.3	1.3	−1.1	1.10	−1.1	−1.4	1.0	−1.6
DESC1 28983	Deleted in HNSCC, putative tumor suppressor gene	−1.2	−7	4	7.7	−4.3	1.400	−26	−1.8	−1.3	2.9	1.6	3	−79	−101	1.2	−3	5.8	−2.7	−1.2	−1.7
SPPR3 6707	Cornified envelope precursor, necessary for epithelial terminal differentiation	−1.4	ND (−1.1)	5.2	42.5 (52)	−3.3 (ND)	−3	ND	−2.5	ND	3	3.3	ND (3.3)	−17.6	−12	1.1	−3.2	ND	−2.1	−3.9	−3.3
UBD 10537	Functions in ubiquitinization of proteins and associated with apoptosis	1.6	370 (55.7)	−1.4	3.6 (2.5)	41 (9.2)	−5	−1.5	2.4	−2.5	−1.6	7.3	1.3 (1.1)	1.5	1.2	−5	−2	−2.5	1.1	−7.4	−1.6
iNOS 4843	High output NOS isoform	−2.9	−20	−1.6	−5.2	1	−3.5	−8.2	5.5	3.7	−1.4	1.90	2.4	1.1	3	−1.4	1	−2.9	−1.2	−3.8	−1.0
VEGF 7422	Angiogenic cytokine that initiates all steps necessary for angiogenesis	3.7	−10	1.2	1	1.7	−5.4	−1.2	−1.9	−2.1	1.8	−1.7	−3	2.4	−1.1	−2.5	−2.5	−1.2	−1.8	−1.8	−1.0
COX-2 4513	High output COX isoform	1.3	−1.8	−4.4	0.7	164	−3364	−1.6	1.4	−1.4	−2.3	1.4	14.2	2.7	0.7	−1.6	−4.9	2.2	−4.6	−1.8	−2.4
TGM1 7051	Keratinocyte-specific transglutaminase, functions in cornified envelope formation	1.5	−6.6	1.7	−3.8	−1.5	−1.1	−2.1	2.5	−1.1	3.9	5.9	−9.4	−6.2	−2.5	−93	−1.3	−1.1	−1.8	1.1	1.6
INV3713	Protein necessary for keratinocyte cornified envelope formation	−1	−1.2	−1.2	−1.3	−3.3	−2.1	−1.4	1.2	−1.2	−1.1	2.3	−.2	−1.1	1.1	−5.1	−1.8	3.700	−2.5	−1.2	10.7
LOR 4014	Protein associated with keratinocyte terminal differentiation	1.1	30	−59	39045	−5.2	−10.6	2.2	−1.3	91533	−1.1	−4	3.1	630	143	1519	−4.3	−92	−10	−59	66.9
KER13 3860	One of two paired keratins in suprabasilar masticatory epithelium	−7.4	5.8	−5.8	−2.9	−26	−1.9	1.4	1.1	2.2	1.6	−2	−1.1	26032	2429	−299	1.5	−159	−1.2	26.6	13.6
SPRR2C 6702	Cornified envelope precursor	−1.3	2.2	4	1.8	−1.2	−1.6	−2.2	5.4	1.3	−1.5	2	3.4	−3.5	−9.5	−40	−1.4	−1.3	3.8	−2.3	−2.0

NOTE: Data expressed as fold change in the posttreatment tissues relative to participant-matched pretreatment tissues. Values in parentheses are fold change data obtained by microarray analyses (patient nos. 2, 5, 6, and 13).

Table 4

Summarized data from black raspberry gel clinical trials

Patient no.	RT-PCR changes ≥2-fold in a therapeutic direction (16 genes analyzed)	IEN epithelial COX-2 protein	IEN epithelial NOS protein	Effect of treatment on histopathology	Reversal(s) of LOH*	Change in MVD (%)	Overall therapeutic responses (%)
1	1	Decrease (−40.0%)	Decrease (−13.1%)	Decrease (1 grade)	4 of 4	Not conducted†	8 of 23 (34.8)
2	9	Decrease (−4.0%)	Decrease (−27.7%)	Decrease (1 grade)	0 of 1	−67.3	12 of 20 (60.0)[a]
3	4	Decrease (−19%)	Decrease (−11.0)	Decrease (1 grade)	0 of 1	−4.9	7 of 20 (35.0)[b]
5	5	Increase (+3.0%)	Increase (+42.3%)	Decrease (1 grade)	0 of 2	Not conducted†	6 of 21 (28.6)
6	3	Decrease (−3.0%)	Decrease (−29.0%)	No change	0 of 0	Not conducted†	5 of 19 (26.3)
7	5	Decrease (−24.0%)	Increase (+22.7%)	No change	3 of 6	−38.4	9 of 25 (36.0)[c]
8	5	Decrease (−43.0%)	Decrease (−32.8%)	Increase (3 grades)	2 of 11	Not conducted†	9 of 30 (30.0)
9	6	Decrease (−30.0%)	Decrease (−24.1%)	Decrease (1 grade)	0 of 0	Not conducted†	9 of 19 (47.4)
10	3	Decrease (−1.2%)	Decrease (−3.0%)	Decrease (2 grades)	5 of 6	−2.9	11 of 25 (44.0)[d]
11	5	Decrease (−26.0%)	Increase (+17.6%)	Increase (1 grade)	2 of 8	Not conducted†	8 of 27 (29.6)
12	8	Increase (+0.4%)	Decrease (−16.0%)	No change	1 of 2	Not conducted†	10 of 21 (47.6)
13	6	Decrease (−19.0%)	Increase (+13.0%)	No change	1 of 2	+44.1	8 of 21 (38.1)[h]
14	5	Decrease (−18.0%)	Decrease (−8.2%)	Decrease (2 grades)	0 of 0	−4.4	8 of 19 (42.1)[e]
15	4	Increase (+83.0%)	Increase (+11.8%)	No change	0 of 0	−28.9	4 of 19 (21.1)[f]
17	2	Decrease (−38.3%)	Increase (+9.7%)	Increase	2 of 4	Not conducted†	5 of 23 (21.7)
18	4	Decrease (−76.1%)	Decrease (−29.0%)	No change	0 of 4	+10.1	6 of 23 (26.1)[i]
19	4	Decrease (−51.7%)	Decrease (−90.0%)	Increase (1 grade)	0 of 0	+7.7	6 of 19 (31.6)[j]
20	4	Decrease (−88.9%)	Decrease (−38.0%)	No change	Not conducted†	−19.8	6 of 19 (31.6)[g]
21	4	Decrease (−85.0%)	Increase (+83.0%)	No change	Not conducted†	+12.0	5 of 19 (26.3)[k]
22	5	Decrease (−25.2%)	Increase (+177.0%)	No change	Not conducted†	Not conducted†	6 of 19 (31.6)
		Mean = −25.3% ± 8.3 SE ($P < 0.005$)	Mean = 2.8% ± 12.1 SE ($P = 0.53$)	Decrease = 35%			High (>45%) = 3
		Decrease = 85%	Decrease = 60%	Increase = 20%			Mid (>30–44%) = 9
		Increase = 15%	Increase = 40%	Stable = 45%			Low (≤30%) = 8

[*] Shumway et al. (31).

[†] Not conducted due to insufficient tissue.