Topical Application of a Mucoadhesive Freeze-Dried Black Raspberry Gel Induces Clinical and Histologic Regression and Reduces Loss of Heterozygosity Events in Premalignant Oral Intraepithelial Lesions: Results from a Multicentered, Placebo-Controlled Clinical Trial

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Abstract

Purpose: Approximately 30% higher grade premalignant oral intraepithelial neoplasia (OIN) lesions will progress to oral cancer. Although surgery is the OIN treatment mainstay, many OIN lesions recur, which is highly problematic for both surgeons and patients. This clinical trial assessed the chemopreventive efficacy of a natural product-based bioadhesive gel on OIN lesions.

Experimental Design: This placebo-controlled multicenter study investigated the effects of topical application of bioadhesive gels that contained either 10% w/w freeze-dried black raspberries (BRB) or an identical formulation devoid of BRB placebo to biopsy-confirmed OIN lesions (0.5 g/C2 q.i.d., 12 weeks). Baseline evaluative parameters (size, histologic grade, LOH events) were comparable in the randomly assigned BRB (n = 22) and placebo (n = 18) gel cohorts. Evaluative parameters were: histologic grade, clinical size, and LOH.

Results: Topical application of the BRB gel to OIN lesions resulted in statistically significant reductions in lesional sizes, histologic grades, and LOH events. In contrast, placebo gel lesions demonstrated a significant increase in lesional size and no significant effects on histologic grade or LOH events. Collectively, these data strongly support BRB’s chemopreventive impact. A cohort of very BRB-responsive patients, as demonstrated by high therapeutic efficacy, was identified. Corresponding protein profiling studies, which demonstrated higher pretreatment levels of BRB metabolic and keratinocyte differentiation enzymes in BRB-responsive lesions, reinforce the importance of local metabolism and differentiation competency.

Conclusions: Results from this trial substantiate the LOH reductions identified in the pilot BRB gel study and extend therapeutic effects to significant improvements in histologic grade and lesional size. Clin Cancer Res; 20(7); 1910–24. ©2014 AACR.

Introduction

Oral squamous cell carcinoma (OSCC) is a worldwide health problem and one of the most challenging-to-treat human malignancies (1). This is due to the insidious nature of its early disease, reliance upon surgery as the primary treatment modality, and the difficulty of achieving locoregional disease control (1, 2). Many patients with OSCC die from massive local recurrence or second primary tumors (3, 4). Also, despite treatment innovations like inductive chemotherapy and radiation intensification programs, OSCC survival rates remain among the lowest for solid tumors (4, 5). Those patients fortunate enough to be cured often encounter major esthetic and functional issues with their face and mouth (6).

OSCCs arise from malignant progression of a recognized precursor surface epithelial lesion, that is, oral
translational relevance

Oral squamous cell carcinoma (OSCC), which arises from its premalignant precursor oral intraepithelial neoplasia (OIN), is a worldwide health problem. Although not all OIN lesions will transform, approximately 30% of the higher grade lesions progress to OSCC. Furthermore, OIN lesions often recur despite complete surgical excision. Numerous chemoprevention studies have therefore attempted to induce regression in or prevent progression of OIN lesions. Modest success and dose-limiting toxicities were often the outcomes of these clinical trials. The majority of previous OIN clinical studies relied upon systemic chemopreventive agent administration. In contrast, this study assessed the effects of a food-based (freeze-dried black raspberries) bioadhesive gel on OIN lesions. Our results, which demonstrate BRB gel significantly reduces LOH, lesional size, and histologic grade in OIN lesions without any toxicities combined the absence of such effects in the placebo gel cohort, are favorable.

Results from the current study confirm that therapeutic efficacy is attributable to the BRB constituents as only the BRB-containing and a BRB-devoid (placebo) gel and doubled treatment time (3 months vs. 6 weeks). Therapeutic efficacies of BRB-containing and placebo gels (0.5 g applied q.i.d. for 3 months) were determined by their effects on: (i) clinical lesional size, (ii) OIN histopathologic grade, and (iii) LOH status at putative tumor suppressor gene loci associated with OIN progression to OSCC [3p14 (FHIT), 9p21 (INK4a/ARF), 9p22 (IFN-γ), and 17p13 (p53)]. Pre-treatment OIN biopsies were used to microscopically confirm a premalignant diagnosis and provide baseline biomarkers. Additional protein profiling assays were conducted to assess the contribution of differentiation and metabolic-local recycling enzymes on BRB gel efficacy. Results from the current study confirm that therapeutic efficacy is attributable to the BRB constituents as only the BRB gel-treated lesions showed significant therapeutic responses as indicated by improvement in lesional size and histologic grade and reduction of LOH events. In contrast, placebo gel-treated lesions significantly increased in size and did not show significant changes in histologic regression or LOH status over the study duration.

Materials and Methods

Berry gel manufacturing

Freshly harvested BRB from Strums Berry Farm were immediately freeze dried and ground into powder at Oregon Freeze Dry Inc. BRB powder was shipped on dry ice to JR Freeze Dry Inc. BRB powder was immediately freeze dried and ground into powder at Oregon Freeze Dry Inc. BRB powder was shipped on dry ice to JR Freeze Dry Inc. BRB powder was immediately freeze dried and ground into powder at Oregon Freeze Dry Inc. BRB powder was shipped on dry ice to JR Freeze Dry Inc. BRB powder was immediately freeze dried and ground into powder at Oregon Freeze Dry Inc. BRB powder was shipped on dry ice to JR Freeze Dry Inc.
Clinical trial inclusion/exclusion criteria

Investigational New Drug approval was obtained from the U.S. Food and Drug Administration (IND#109774). Institutional Review Board (IRB) approvals were obtained from the IRBs at all three participating Universities, that is, Ohio State, University of North Carolina, Chapel Hill and Louisville (Trial registration ID: NCT01192204). Forty adults (see Table 1) were consented for trial participation. Inclusion criteria were microscopically confirmed premalignant oral epithelial lesions, no use of tobacco products for 6 weeks prior and during the 3-month study, and no previous history of cancer (except for basal cell carcinoma of the skin). Participants were screened before entrance into and during (10–12 days recall intervals) the trial for no tobacco use compliance via unannounced saliva testing for nicotine (NicAlert, JANT Pharmacal Corporation). Trial exclusion criteria were previous or current history of non-basal cell cancer, use of tobacco products, and either a microscopic diagnosis of no premalignant change or OSCC in the pretrial biopsy. The National Cancer Institute (Bethesda, MD) common toxicity criteria version 4.0 was used. All participants returned to clinic every 10 to 14 days for continued assessment. Used gel tubes were returned and new gels were dispensed.

Histologic grading criteria

Formalin-fixed, paraffin-embedded biopsies were sectioned and stained with hematoxylin and eosin. Photomicrographs were taken using an Olympus BX51 microscope (Olympus) with ×10 objective lens and a Nikon DS-Fi1 digital camera (Nikon) through ImagePro 6.2 software (Media Cybernetics). A 0 to 8 grade scale (0 = normal with or without hyperkeratosis, 1 = atypia with crisply defined clinical margins, 2 = mild dysplasia, 3 = mild-moderate dysplasia, 4 = moderate dysplasia, 5 = moderate-severe dysplasia, 6 = severe dysplasia, 7 = carcinoma in situ, 8 = invasive SCC) was used to rank the light microscopic diagnoses. A diagnosis of "hyperkeratosis" alone indicated a benign, reactive change without evidence of premalignant potential. "Atypia" indicated architectural and cytologic alterations that in the clinical setting of an adherent crisply defined white plaque represent early premalignant change. To reduce subjectivity, two board-certified oral and maxillofacial pathologists reached consensus before a final diagnosis was rendered (20). All participating oral pathologists, surgeons, and patients were blinded to the patients' gel assignments.

Assessment of lesional clinical size

Clinical photographs of the participants’ OIN lesions (pretreatment-time 0, week 1 (biopsy follow-up, baseline for subsequent measurements), mid-study (6 weeks), and immediately before final biopsy) were taken with a calibrated measuring device (Puritan) placed parallel to the long axis of the lesion. Acquired clinical images were analyzed using ImagePro 6.2 software (Media Cybernetics). Lesional sizes were normalized to square millimeters (mm²) according to the following formula: lesional size mm² = pixels of lesional area × 100/pixels of 1 centimeter unit on the calibration device in the same image ². The remaining lesional area after the initial biopsy and before gel treatment was the pretreatment size. Posttreatment lesional size was the residual lesional area after 3 months gel treatment and just before the final biopsy.

Tissue microdissection and DNA isolation

Formalin-fixed, paraffin-embedded biopsies were sectioned at 8 µm thickness on PEN membrane slides (Carl Zeiss). The entire oral epithelia and the corresponding histologically normal connective tissue were independently microdissected using the PALM Microbeam IV laser capture microdissection (LCM) system (Carl Zeiss) at The OSU Laser Capture Molecular Core. QIAamp DNA Micro Kit (QIAGEN) was used for DNA extraction.

PCR amplification and detection of allelic imbalance

Genomic DNA isolated from the LCM samples was amplified using the predesigned primers with a 5’ fluorescent label on the forward primer (Life Technologies). The microsatellite markers selected for LOH analyses and their corresponding loci and associated genes were: 3p14 [D3S1007 (VHL), D3S1234 (FHIT)], 9p21 [D9S171, D9S174 (P16/CDKN2A), D9S1751 (P16)], 9p22 (IFN-α), and 17p13 [D17S786 (P53) and TP53]. Twenty microliter PCR mixture, which contained 5 µL of genomic DNA, 2 µL of primer mix (0.5 µmol/L of each primer), 10 µL of AmpliTaq Gold 360 master mix (Life Technologies), and 3 µL of nuclease-free water, was amplified using a iCycler thermal cycler (Bio-Rad). PCR conditions were: 95 °C for 5 minutes followed by 40 cycles of 95 °C for 15 seconds, 59 °C for 30 seconds, 72 °C for 45 seconds, and a final elongation step of 72 °C for 7 minutes. Fragment analyses were performed at the OSU Plant Microbe Genomics Facility using the Applied Biosystems 3730 sequence analyzer. One microliter of PCR product DNA was added to 9 µL HiDi formamide (formamide; Applied Biosystems, Inc.) and 0.2 or 0.4 µL (volume dependent upon DNA concentration) GeneScan-500 LIZ Size Standard (Applied Biosystems, Inc.) for

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Table 1. Cumulative clinical trial data

|   | Sex | Age | Pk y (a) | PVL | Previous biopsies | Lesional size (mm²) | Pretreatment | Posttreatment | Pretreatment | Posttreatment | Cumulative responsiveness (score) | 3-Month recurrence |
|---|-----|-----|----------|-----|------------------|-------------------|---------------|--------------|---------------|--------------|--------------------|------------------|------------------|
| Active: | | | | | | | | | | | | | |
| A1 | F | 55 | 0 | N | Y (5) | 32.07 | 19.68 | DYS (mild to moderate) | DYS (mild) | 3 | 0 | High (5) | Y |
| A2 | F | 62 | 0 | Y | Y (2) | 59.84 | 54.06 | ATY | ATY | 3 | 0 | Intermediate (3) | N |
| A3 | F | 74 | 26 (1991) | Y | Y (3) | 21.54 | 23.27 | ATY | ATY | 1 | 1 | Non (0) | Y |
| A4 | M | 69 | 0 | Y | Y (4) | 8.87 | 6.80 | DYS (focally mild) | DYS (mild) | 2 | 0 | Low (2) | N |
| A5 | F | 65 | 0 | N | Y (6) | 58.18 | 0.00 | DYS (mild) | Normal | 3 | 0 | High (6) | LC |
| A6 | F | 56 | 5 (1980) | N | N | 6.78 | 0.00 | DYS (severe) | DYS (mild to moderate) | 0 | 0 | Low (1) | Y |
| A7 | F | 55 | 0 | N | Y (4) | 30.60 | 33.93 | DYS (mild) | DYS (mild) | 1 | 0 | Low (1) | N |
| A8 | F | 69 | 35 (1996) | Y | Y (3) | 24.07 | 10.88 | ATY | ATY | 0 | 0 | Low (2) | N |
| A9 | M | 60 | 20 (1975) | Y | Y (1) | 170.87 | 72.56 | DYS (mild) | ATY | 0 | 0 | Low (1) | N |
| A10 | M | 66 | 5 (1968) | N | Y (1) | 53.98 | 48.71 | DYS (mild) | ATY | 0 | 0 | Low (2) | N |
| A11 | M | 47 | 0 | Y | N | 63.33 | 106.45 | DYS (mild) | DYS (mild) | 3 | 3 | Low (4) | N |
| A12 | F | 59 | 25 (2010) | Y | Y (1) | 279.24 | 231.40 | DYS (mild) | ATY | 1 | 1 | Low (1) | N |
| A13 | F | 70 | 0 | Y | N | 12.15 | 8.82 | DYS (mild) | ATY | 0 | 0 | Low (1) | N |
| A14 | F | 61 | 0 | Y | Y (1) | 227.70 | 137.69 | ATY | ATY | 0 | 0 | Low (1) | N |
| A15 | F | 70 | 20 (1996) | N | N | 23.85 | 16.99 | DYS (mild to focally severe) | DYS (mild) | 1 | 0 | Intermediate (3) | N |
| A16 | F | 56 | 1.05 (1997) | N | N | 53.28 | 49.44 | ATY | ATY | 3 | 0 | Intermediate (3) | Y |
| A17 | F | 60 | 0 | N | Y (1) | 110.71 | 54.60 | ATY | ORT | 1 | 0 | High (4) | N |
| A18 | F | 66 | 0 | N | Y (1) | 63.37 | 30.05 | DYS (mild to focally severe) | DYS (mild) | 2 | 2 | Intermediate (3) | N |
| A19 | M | 77 | 0 | Y | Y (1) | 75.06 | 77.99 | DYS (ulcerated severe) | DYS (severe) | 4 | 1 | Intermediate (3) | N |
| Active: | | | | | | | | | | | | | |
| Placebo: | | | | | | | | | | | | | |
| P1 | M | 78 | 5 (1992) | Y | Y (4) | 25.55 | 46.44 | DYS (mild to moderate) | DYS (mild to moderate) | 2 | 3 | Non (–4) | Y |
| P2 | M | 63 | 0 | N | Y (3) | 8.76 | 5.94 | DYS (mild to severe) | DYS (mild to moderate) | 1 | 1 | Low (1) | Y |
| P3 | M | 60 | 0 | N | Y (1) | 50.49 | 51.38 | DYS (mild to focaly moderate) | DYS (mild) | 0 | 0 | Low (1) | Y |

(Continued on the following page)
Table 1. Cumulative clinical trial data (Cont’d)

<table>
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<th>#</th>
<th>Sex</th>
<th>Age</th>
<th>Pk y (a)</th>
<th>PVL</th>
<th>Previous biopsies b</th>
<th>Lesional size (mm²)</th>
<th>Pretreatment</th>
<th>Histopathology</th>
<th>LOH</th>
<th>Cumulative responsiveness (score)</th>
<th>3-Month recurrence</th>
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<td>N</td>
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<tr>
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<td>0</td>
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<td>DYS (Focally mild)</td>
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<tr>
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<td>N</td>
<td>9.81 10.96</td>
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<td>DYS (Mild)</td>
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<td>0</td>
<td>Non (0)</td>
</tr>
<tr>
<td>P8</td>
<td>F</td>
<td>32</td>
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<td>Y (8)</td>
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<td>DYS (Mild)</td>
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<td>2</td>
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<tr>
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<td>N</td>
<td>N</td>
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<td>Y (1)</td>
<td>NE  NE</td>
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<td>F</td>
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<td>10.62 8.75</td>
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<td>DYS (Moderate to severe)</td>
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<td>P14</td>
<td>F</td>
<td>68</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>77.87 74.58</td>
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<td>95.45 123.13</td>
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<td>107.23 167.23</td>
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<td>DYS (Mild)</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

Abbreviations: ATY, atypia; DYS, dysplasia; LC, lost contact; NE, not evaluable; ORT, orthokeratosis; Pk y, pack years; PVL, proliferative verrucous leukoplakia.

a(Year) patient quit smoking.

bPrevious biopsies for OIN at lesional treatment site (number of biopsies performed before the trial).
analysis. Both automatic and manual (enabled editing) settings for allele identification (GeneMapper v4.0, Applied Biosystems) were used to analyze electropherogram data. Peak intensities ≤50 RFUs (relative fluorescent units) were excluded for being within background. Microsatellite marker peak patterns and allele sizes were established from normal DNA (15). Connective tissue control samples with only one allele were deemed “not interpretable” (NI). In several instances, the PCR amplification products in the normal connective tissue for a particular patient/marker combination were inadequate to allow LOH determination and were designated as “not available” (NA). LOH determinations were made using a modification of the protocol established by Canzian and colleagues (21) as described by Shumway and colleagues (15), using an increased level of stringency (>50% reduction in peak intensity) to accept the presence of LOH (21). Baseline OIN LOH status was determined from the initial biopsy and then compared with LOH status in treated lesional tissue.

**Evaluation of differentiation and local enteric recycling enzymes and cornified envelope precursor proteins by immunoblot analyses**

Western immunoblotting was conducted as previously described (19). Primary antibodies and dilutions were: transglutaminase 1 (TGase 1, Abcam, ab27000, 1:400), loricrin (Abcam, ab85679, 1 μg/mL, involucrin (Santa Cruz Biotechnology, sc-21748, 1:200), cytokeratin 10/13 (Santa Cruz Biotechnology, sc-70908, 1:200), UDP-glucuronosyltransferase 1A (UGT1A, Santa Cruz Biotechnology, sc25847, 1–400), UDP glucose dehydrogenase (UDP-GlcDH, Santa Cruz Biotechnology, sc137057, 1:100), pan-cytokeratin (Santa Cruz Biotechnology, sc8018, 1:200). Secondary antibodies were: goat anti-mouse IgG-HRP (Dako). Positive controls for TGase1 (TGase 293T lysate), involucrin (CCD-1064 cell lysate), loricrin (Hep G2 cell lysate), cytokeratin 10/13 (A-431 whole-cell lysate), pancytokeratin (A-431 whole-cell lysate), UGT1A (Hep G2 cell lysate), and UDP-GlcDH (Hep G2 cell lysate) were all purchased from Santa Cruz Biotechnology. The Kodak 1D3 image analysis software (Kodak) was used for densitometry analyses. Results were normalized relative to endogenous pancytokeratin expression because: (i) the enzymes either exclusively (transglutaminase 1) or predominantly reside in surface epithelia (19), (ii) cornified envelope proteins are epithelial, and (iii) proteins normalized relative to epithelial as opposed to epithelia + connective tissue content of the biopsies. See Supplementary Table S1 for the physiological functions of these selected proteins.

**Determination of the overall therapeutic responsiveness**

A responsiveness score that incorporated extent of changes in lesional size, histologic grade, and LOH was determined for every trial participant. A −3 to 3 responsiveness score scale of lesional size was developed to reflect the extent of change in lesional size. That is, ≥75% decrease = 3, 50% to 74% decrease = 2, 25% to 49% decrease = 1, 0% to 24% decrease or increase = 0, 25% to 49% increase = −1, 50% to 74% increase = −2, and ≥75% increase = −3. The cumulative responsiveness score was then calculated according to the following formula: cumulative responsiveness score = lesional size responsiveness score + histologic grade responsiveness score (pretreatment grade − posttreatment grade) + LOH responsiveness score (pretreatment events − posttreatment events). Finally, overall therapeutic responsiveness was categorized in accordance with cumulative scores as follows: (i) high responder ≥ 4, (ii) intermediate responder = 3, (iii) low responder = 1 or 2, and (iv) nonresponder ≤ 0.

**Statistical analyses**

Two-tailed Mann–Whitney U tests were used to compare pretreatment baseline parameters, that is, size, histologic grade and LOH events in the BRB and placebo gel cohorts. A Wilcoxon matched-pairs signed rank test was used to compare the pre and posttreatment histologic grades, clinical lesional sizes, and LOH events. Cumulative treatment responses (effects on lesional size, histologic grade, and LOH events) were evaluated by two-tailed unpaired t test. A Fisher exact test was used to compare distribution of responsiveness between the BRB and placebo gel treatments. Associations between two therapeutic evaluative parameters were determined by a Spearman rank correlation. Relationships among size, histologic grade, and LOH used multiple regression analysis. Normality of data determined the use of parametric versus nonparametric analyses.

**Results**

**Patient demographics and comparable pretreatment baseline parameters**

Forty patients participated in this study. Participants’ ages ranged from 44 to 77 years (mean 62.2 ± 1.8) and 32 to 78 years (mean 57.7 ± 2.9) in the BRB and placebo gel cohorts, respectively. A majority of the patients never smoked (55% in BRB and 67% placebo gels) and tongue was the most common OIN site in both groups. The gender distribution in the BRB gel cohort was 78.2% (15 women) and 31.8% (7 men), whereas the placebo group was evenly distributed (50% each, 9 women, 9 men; Table 1). Thirty of the participants had OIN lesions (16 in BRB-72.7% and 14 in the placebo-77.7%) that were recalcitrant to surgery, and had recurred multiple times (2–8) at the same site before trial participation. Twelve BRB (54.5%) and three placebo gel (16.7%) participants had a history of multiple premalignant oral epithelial lesions dispersed throughout their mouth, consistent with a diagnosis of proliferative verrucous leukoplakia (Table 1). No histopathologic evidence of concurrent human papillomavirus infection, for example, koilocytic change in lesional epithelial cells was observed in any of the pre or posttreatment lesional tissue biopsies.

Patients in the BRB and placebo gel cohorts had comparable pretreatment ages, clinical lesional sizes, histologic grades, and LOH status. BRB gel patients’ pretreatment lesional sizes (70.95 ± 15.66 mm², n = 21) were

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comparable with placebo patients’ lesions (61.63 ± 14.39 mm², n = 17; Fig. 1C). One patient in both groups (subjects A4 and P11) had diffuse confluent adherent white plaques that prohibited delineation of a discreetly measurable lesional site. Comparable pretreatment histologic grades were present in both groups (pretreatment BRB group 2.36 ± 0.35, n = 22) and pretreatment placebo group (2.83 ± 0.34, n = 18); see Fig. 2C]. Pretreatment LOH events (per patient) for all eight markers were also comparable between the BRB gel group (1.36 ± 0.28, n = 22) and the placebo gel group (1.06 ± 0.24, n = 18; Fig. 3C). Forty three percent of BRB and 26% of placebo pretreatment lesions demonstrated 9p associated allelic imbalance. The overall pretreatment LOH indices were 27.8% and 21.8%, in the BRB and placebo gels, respectively.

No deleterious effects were observed in either the BRB gel or placebo gel cohorts and compliance was excellent

Although topical application of a gel could result in deleterious effects, for example, contact mucositis or superimposed Candidiasis, no participant experienced any treatment-associated complications. Furthermore, as determined by the minimal residual gel in the returned gel tubes (>95% dose used), patient compliance was high.

BRB gel significantly decreased OIN lesional clinical size

Following 3 months of BRB gel application, 16 of the 21 BRB-treated lesions decreased in size (P = 0.0019) for an average overall size decrease of 26%. In contrast, 17 of the 19 placebo gel-treated lesions increased in clinical lesional size (P = 0.0395) with an average increase of 18% (see Table 1; Fig. 1A–C). Although none of the placebo gel patients experienced complete lesion regression, two BRB gel patients had 100% lesional resolution. These individuals’ mucosa returned to a clinically (and histologically) normal and healthy appearance (Fig. 1D).

OIN histologic grade was also significantly reduced by BRB gel application, whereas placebo gel had no significant effect

Comparison of the histologic grade of the pretreatment versus posttreatment lesional tissue biopsies demonstrated that application of the BRB gel resulted in a statistical decrease in histopathologic grade (P = 0.0488), whereas placebo gel application did not significantly impact histopathologic grade (P = 0.4961; Table 1; Fig. 2).

Topical application of BRB gel significantly reduced allelic imbalance in OIN lesions

BRB gel-treated lesions demonstrated a statistically significant reduction in LOH events at all 9p loci relative to pretreatment parameters P = 0.016, n = 22. With regard to all loci evaluated, BRB gel treatment significantly reduced overall LOH events, P = 0.002, n = 22. In contrast, placebo gel application resulted in comparable 9p pre and posttreatment LOH status and did not significantly reduce overall LOH events (Table 1; Fig. 3).

Collective assessment of extent of treatment effects on lesional size, histopathologic grade, and LOH events reveals highly BRB-responsive cohort

Cumulative scores, which reflected the extent of gel application effect on lesional size, histologic grade, and LOH indices, were assigned for every trial participant (Table 1; Fig. 4A and B). BRB gel participants had a statistically significant (P = 0.004) higher scores, indicative of greater therapeutic effects (Fig. 4C). Nine of the 22 BRB gel participants’ (41%) OIN lesions achieved high to intermediate responsiveness, whereas all of the placebo patients were either low (55%) or nonresponders (no therapeutic effects; Table 1; Fig. 4D). Correlative analyses showed a significant association between improvement in histologic grade and reduction in lesional size (P = 0.009) in the BRB gel treatment cohort (Fig. 4E). Multivariate analyses of the BRB gel OIN data also revealed a significant relationship among treatment effects on lesional size (identified as outcome), histologic grade, and LOH indices (P = 0.0001). Consistent with the Spearman correlation findings, histologic grade made the largest contribution to the multivariate significance. No linear associations or multivariate relationships were detected in the placebo gel data.

Baseline levels of differentiation and enteric recycling proteins may help identify BRB-responsive OIN lesions

Samples for protein assessment were only available from the larger OIN lesions, resulting in a reduced dataset. Baseline intra-BRB gel cohort analyses reveal a trend for higher lesional intraepithelial levels of differentiation-associated proteins (T-GCase 1, involucrin 140 kDa, involucrin 68 kDa, loricin and cytokeratin 10/13) and enteric recycling enzymes (UGT1A and UDP-GlcDH) in those OIN lesions which responded in a therapeutic fashion to BRB gel application (Fig. 5A and B). No significant associations were identified in the placebo gel cohort. (Fig. 5C and D). Finally, the most BRB gel-responsive OIN lesions had the highest pretreatment levels of differentiation-associated proteins (Supplementary Fig. S1).

Longer term follow-up reveals OIN lesional recurrence in both cohorts

Thirty eight of the 40 trial patients were available for the requisite 3-month posttrial evaluation; one patient each was lost to follow up in the BRB and placebo gel cohorts. Three months after trial cessation, 6 of 22 BRB and 7 of 17 placebo patients had visible evidence of lesional recurrence at the former treatment sites (Table 1). Only one patient (P6) lesion had a clinically significant lesional recurrence that merited biopsy scheduling at the 3-month recall. Patients were then returned to their previous oral health care providers for subsequent care. As many of these patients are treated by oral maxillofacial surgeons who use the pathology biopsy services at the trial institutions, longer term follow-up (4 to 31 months postfinal study biopsy) was available for many trial participants (see Supplementary Table S2). Longer term poststudy biopsies were received from 8 of the 22 BRB gel and 6 of the 18 placebo gel cohort.
patients. Two BRB gel cohort patients’ lesions (25%) regressed to nonpremalignant states, four biopsies remained stable (50%), and two lesions progressed (Supplementary Table S2). One of these progressive patients underwent progression of two histologic grades, whereas another lesion returned to its pretrial histopathologic grade.

Figure 1. Effect of gel treatment on lesional size. Histograms A (BRB gel) and B (placebo gel) depict pre- and posttreatment lesional size (mm²) of individual subjects’ OIN lesions. The lesional sizes of subject A4 in the BRB group and subject P11 in the placebo group were not included as the extensive distribution and confluent nature of their dysplastic lesions made accurate measurement impossible. C, the mean pretreatment lesional sizes of both groups were statistically comparable. Intragroup pre/post lesional sizes significantly decreased in the BRB gel treatment group, whereas significantly increased in the placebo group. D, intergroup comparison reviewed a significant difference between the BRB and placebo groups. E and F, this clinical photograph of subject A6 was taken before the initial biopsy of the crisply defined rhomboid white plaque close to commissure. The area of the residual lesion remained after initial biopsy was measured as the pretreatment biopsy size. F, clinical photograph at the same site after 3 months BRB gel treatment and immediately before the final biopsy. The remaining lesion had completely regressed and the buccal mucosa at the treatment site had a normal clinical appearance.
Changes in histologic grade (BRB group)

Changes in histologic grade

BRB Placebo

Pretreatment

Posttreatment

Histologic grade (mean ± SEM)

Number of subjects

A

B

C

D

E

F

G

H

Histologic grade

↓ 19.06%
P = 0.0488*

↓ 5.65%
P = 0.4961*

↓ 0.65%
P = 0.4961*

* Wilcoxon matched-pairs signed rank test
† Mann–Whitney U two-tailed unpaired test


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Three of the six placebo gel patients' lesions remained stable, three lesions progressed. Two of the three progressive lesions from the placebo gel cohort increased one histologic grade, whereas the third individual's lesion (P11) progressed from atypia (pretreatment) to moderate dysplasia (postplacebo gel application) to OSCC in the 12 months posttrial (Supplementary Table S2).

Discussion

Topical application of a 10% BRB gel resulted in significant reduction in size, histologic grade, and LOH events in OIN lesions. The absence of comparable results following placebo gel application strongly supports BRB's chemopreventive impact and dispels other contributions such as topical stimulation during gel application or hydrogel constituents.

Much of BRB's chemopreventive effect is derived from the primary phenolic compounds, that is, anthocyanins (16, 22, 23). BRB anthocyanins are redox active compounds which possess both redox scavenging and redox generating properties (24, 25). Accordingly, anthocyanins can quench reactive oxygen species (ROS)-mediated signaling, reduce adverse DNA–ROS and/or protein–ROS interactions, and inhibit lipid peroxidation (26). Such activities can ultimately suppress inappropriately sustained proliferation and limit DNA and protein perturbations (23, 27). As bulky sugar moieties reduce ROS scavenging capacity, epithelial and oral microflora-initiated deglycosylation generates superior antioxidants, that is, the labile aglycones or the more stable protocatechuic acid (28, 29). Anthocyanins also generate ROS (25). At alcalotic pH levels, anthocyanins/anthocyanidins are speculated to form quinones, release superoxide and H2O2, and in the presence of oxidized transition metals, and generate the highly mutagenic hydroxyl radical (25). Proximity to anthocyanin-generated superoxide anions and quinone reduction can induce mitochondrial uncoupling, initiate mitochondrial failure, and trigger apoptosis (30, 31). Provided the complexity of factors that can modulate BRB chemopreventive impact, for example, metabolism and recycling, pH, presence of reducing equivalents, and keratinocyte levels of cytoprotective enzymes, the variability in BRB gel responsiveness observed in this study is understandable (14, 15, 19, and 26).

Lesion regression is one of the most consistently used therapeutic efficacy parameter in human clinical trials (10–12). Placebo gel application resulted in an overall size increase in 70.6% lesions, a finding that is consistent with the proliferative potential of nontreated premalignant oral lesions (32). In contrast, BRB gel application reduced size in 76.2% of OIN lesions. These data compare favorably with previous OIN studies (data expressed as % of lesions which showed a decrease in size) which evaluated: Trial 1, 13cis retinoic acid [0.5 mg/kg P.O. × 1 year followed by 0.25 mg/kg P.O. × 2 years (48.1%)], or β-carotene + retinyl palmitate [50 mg/day β-carotene + 25,000 U/day retinyl palmitate for 3 years (42.9%)], or retinyl palmitate [25,000 U/day for long-term follow up (20.0%); ref. 10]; Trial 2, celecoxib (100 mg;41.2%, 200 mg-20%, b.i.d. dosing × 3 months; ref. 12); and Trial 3, green tea extract [combined green tea extract doses 50%, n = 28 (500 mg/m², 750 mg/m², 1,000 mg/m², all doses administered t.i.d. × 3 months; ref. 11]. The corresponding placebo data showed 33% and 18.2% of lesions with size reduction in the celecoxib and green tea extracts, respectively (11, 12). The cis-retinoic acid study did not include a placebo group. Adverse effects accompanied the systemic administration and included grade 3 toxicity with use of 13 cis-retinoic acid and induction of caffeine-attributable insomnia and anxiety with the higher green tea extract doses (10, 11).

Following BRB gel application, 41% of participants achieved a decrease in lesional grade; 4.5% a lesional grade increase, and 54.5% retained stable OIN histology. Although the 41% grade decrease is identical to our pilot BRB gel trial (14), the percentage of OIN lesions that histologically progressed are 5-fold lower in the current study; findings that likely reflect the doubled treatment time of the current trial (14). A range of histologic responsiveness has been observed in other OIN clinical trials (10–13). The "most responsive" group (13 cis-retinoic acid) in the combination 13 cis-retinoic acid, β-carotene, and retinyl palmitate trial demonstrated a 30% histologic reduction (10). In the green tea extract study, 33% (3 of 9) obtained histologic regression in the most responsive but toxicity-associated highest dosing group (1,000 mg/m² t.i.d.), with an overall, a 21.4% rate of histologic regression (6 of 28; ref. 11). The celecoxib trial did not include histopathologic assessment (12). Recently, the effects of combined administration of celecoxib (400 mg b.i.d) with escalating doses (50, 75, 100 mg q.d.) of the EGFR inhibitor erlotinib were assessed on premalignant oral and laryngeal lesions (13). Comparison of baseline histology to final biopsies (obtained at 3, 6, or 12 months) in the 7 evaluable patients showed 43% complete regression (3/7, one laryngeal, two oral lesions), 14% (1/7) partial regression, 29% (2/7) stable disease, and 14% progression (1/7; ref. 13). No placebo group was included (13). Complementary biomarker studies demonstrated significant reduction of EGFR and p-ERK in biopsies that showed...
Figure 3. Effect of gel treatment on LOH. A and B, pre- and posttreatment detected LOH events of individual subjects in BRB gel and placebo gel groups, respectively. C, intragroup and intergroup statistical analyses. Mean LOH events significantly decreased in the BRB gel treatment group, whereas changes in the placebo group were statistically insignificant. The baseline LOH events of both pretreatment groups were statistically comparable. D, classification of subjects as per treatment effects on LOH events. E and F, representative genotyping data depict an LOH event occurred on marker D9S171 in subject A19’s pretreatment samples (loss of one allele (at 154) in the epithelium tissue (E), compared with the two alleles (at 154 and at 162) in the patient’s matched normal connective tissue (F)). G and H, the lost allele (at 154) of D9S171 was recovered in the epithelial tissue (G) of the same patient after 3 months of BRB gel treatment, and the ratio of peak heights in epithelium (G) was comparable with the connective tissue sample (H).
histologic improvement (13). Erlotinib dose escalation was accompanied by toxicities including oral mucositis, rash, anemia, sepsis, and elevated liver enzymes; effects that the authors acknowledged as unfavorable for primary chemoprevention (13). Because oral dysplastic lesions have been shown to be less treatment responsive than oropharyngeal
lesions (33), separate reporting of oral and laryngeal lesions seems warranted in more definitive studies (33).

Because of the effects of first pass metabolism and the need for the agent to perfuse from the connective tissue papilla to the avascular epithelia, bioavailability is often challenging for systemically administered OIN chemopreventives (34). Attempts to address the bioavailability challenge by dose escalation are often accompanied by toxicity (11, 13). Furthermore, it is interesting that neither this recent pharmacokinetic study (13) nor any of the previously cited OIN trials determined the levels of parent compound(s) and/or metabolites achieved at the treatment site (10–12). Before conducting our pilot clinical trial, we established that topical BRB gel application provides a pharmacologic advantage at human oral mucosa (35).

LOH-mediated inactivation of one of the two alleles of tumor suppressor genes followed by silencing of the second allele via promoter methylation or point mutation is a putative and probable tumorigenic mechanism (36, 37). Clinical data, which demonstrate a higher risk of malignant transformation in OIN lesions that harbor LOH at tumor suppressor gene loci, support this premise (15, 38). Pre-treatment LOH events detected in the current study were lower than those detected in either our pilot trial and in two investigations conducted by Mao and colleagues (39, 40). These variations likely reflect differences in baseline lesion histology, LOH analytical methods, and microsatellite markers evaluated (15, 39, 40). Consistent with previous investigations (15, 37, 39, 40), allelic imbalances in this current study were lower than those detected in either our pilot trial and in two investigations conducted by Mao and colleagues (39, 40). BRB gel treatment significantly reduced allelic imbalances, whereas placebo gel did not significantly affect LOH status. To our knowledge, our pilot BRB pilot gel trial (15) and this current study are the only OIN chemoprevention trials to demonstrate a significant reduction in LOH occurrence. We speculate that these data reflect removal of LOH harboring keratinocytes from the proliferative pool via BRB-mediated induction of apoptosis and/or differentiation, as previously demonstrated in vitro studies (31).

Although preliminary, our protein profiling data suggest that BRB gel responsiveness may be associated with lesional keratinocytes’ differentiation and local enteric recycling.
enzymatic capacities. Provided additional studies substantiate these findings, baseline protein levels could be used in a "personalized medicine approach" to identify OIN lesions with a high probability of responsiveness. Notably, the highest pretreatment levels of differentiation-associated proteins were detected in the most responsive BRB gel cohort.

The observed posttreatment lesional recurrences were not surprising, particularly because more than 70% of the patients enrolled in this trial had histories of multiple recurrences of the OIN lesion selected for treatment. These recurrences, which are consistent with retention of genetically altered, long-lived stem cells at the lesional site, emphasize the need for effective, nontoxic, long-term chemoprevention strategies. This study shares shortcomings with other OIN trials. First is the dynamic nature of OIN lesions. OIN lesions with a homogenous clinical appearance can still demonstrate molecular and/or histologic heterogeneity (41). Also, as a result of ongoing epithelial turnover baseline to posttreatment biopsies are not direct comparisons. Instead, these measurements assess the effects of treatment on transient amplifying and more mature lesional keratinocytes over time. Furthermore, low patient numbers precluded our ability to determine whether or not clinical site or baseline histologic grade affected therapeutic responsiveness. Another common clinical trial challenge is the intermittent variation in responsiveness. Our data imply that these results reflect differences in local tissue absorption and the extensive variability in human oral mucosal metabolic bioactivation, local enteric recycling, and keratinocyte differentiation-associated enzymes (19).

A recent editorial by a well-respected oral cancer chemoprevention researcher helps to place our results in perspective (42). In this context, BRB gel outcomes, which include significant reduction in OIN LOH events, lesional size, and histologic grade without adverse effects, are favorable.

Disclosure of Potential Conflicts of Interest
S.R. Mallery has ownership interest (including patents) in BER gel patent. No potential conflicts of interest were disclosed by the other authors.

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References


Topical Application of a Mucoadhesive Freeze-Dried Black Raspberry Gel Induces Clinical and Histologic Regression and Reduces Loss of Heterozygosity Events in Premalignant Oral Intraepithelial Lesions: Results from a Multicentered, Placebo-Controlled Clinical Trial

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