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LETTER

Cellular signaling in sinecatechins-treated external genital and perianal warts: unraveling the mechanism of action of a botanical therapy

Dear Editor,

Derived from green tea leaves, sinecatechins (Veregen®) ointment, 15% is a topical therapy that is FDA-approved to treat human papillomavirus (HPV)-induced external genital and perianal warts (EGW) in immunocompetent patients aged 18 years and older. In two phase 3 trials enrolling over 1,000 participants with EGW, a 16-week treatment regimen with sinecatechins ointment resulted in higher rates of complete clearance of all warts — both baseline and newly emerging warts — when compared to vehicle-treated patients (54.9% vs. 35.4%, respectively; $p < 0.001$) (Tatti et al., 2010). This finding, taken together with the low recurrence rates (6.8%, $p < 0.001$), represented a practical improvement over previous clinical trials with other EGW therapies (Tyring, 2012)

Despite its apparent clinical efficacy, the mechanism of action of sinecatechins in the treatment of EGW is largely unknown. Utilizing *in vitro* biochemical assays, sinecatechins was found to mediate dose-dependent inhibition of several inflammatory proteases, kinases, and oxygenases that have been implicated in the pathogenesis of HPV-induced EGW formation (Tyring, 2012). To this end, other therapeutic mechanisms that have been associated with green tea components include pro-apoptotic, anti-viral, anti-oxidative, anti-angiogenic, and immunostimulatory processes; as a result, it is likely that the mechanism of action specific to sinecatechins-treated EGW is multifactorial. Recently, we published our findings from the first *in vivo* study on this topic, demonstrating that sinecatechins are capable of down-regulating antiapoptotic genes in the NF-κB pathway (Nguyen et al., 2014). While our data confirmed previous *in vitro* findings (Tyring, 2012), this report focused solely on apoptotic gene expressions responsive to sinecatechins treatment. In order to evaluate other key cellular mechanisms contributing to pathogenesis of EGW treated with sinecatechins, we sought to perform additional molecular studies on tissues obtained during the clinical study described previously (Nguyen et al., 2014). In this present report, we selected microarrays designed to provide general yet exhaustive assessment of salient genes involved in pro-inflammatory cellular signaling.

Briefly, the open-label, single-site clinical study enrolled 18 immunocompetent subjects with a clinical diagnosis of EGW and employed specialized microarrays to determine the expression level changes of EGW before and after sinecatechins treatment. Veregen® ointment, 15% was dispensed to patients applied to the target warts 3 times daily for 16 weeks. Three 2-mm punch biopsies were obtained from each patient; the first biopsy was excised at baseline (B1), the second at the first visit with 50% or more clearance of target warts (B2), and the third at the first visit with complete clearance of target lesions (B3).

HPV types from extracted DNA samples were detected by a nested PCR approach as previously described (Fuessel-Haws et al., 2004), and copy number was determined with a custom-made real time PCR kit (Quantification of HPV6_15979, L1 protein, L1 gene, PrimerDesign Ltd). Subjects were classified based on their response to treatment, which was quantitatively determined by measuring change in viral copy number between B1 and B3. Subjects were classified as virological responders (VR) if viral copy number decreased by at least 60% from B1 and were classified as virological non-responders (VNR) if viral copy number remained the same or increased from B1. Of the 18 participants, seven were found to be VR, and 11 were defined to be VNR.

Following cDNA synthesis, analyses of signal transduction and cell cycle gene expressions were performed on TaqMan array 96-well plates utilizing StepOnePlus™ Real-Time PCR System (Life Technologies). Quantification of group-based responses for each gene was determined by calculating a fold change from biopsy 1 to biopsy 2 (B1_2), from biopsy 1 to biopsy 3 (B1_3) and from biopsy 2 to biopsy 3 (B2_3). Gene expression changes were categorized as biologically significant if there was at least a two-fold change, and statistical significance of these fold changes were evaluated using a non-parametric two-sided Wilcoxon signed-rank test

Table 1. Comparison between biopsy groups (B1, B2, B3) in virological responders (VR) and virological non-responders (VNR).

Gene	Response	B1 to B2		B1 to B3		B2 to B3	
		RQ	p-value	RQ	p-value	RQ	p-value
BIRC3	VR	0.23901	0.0313*	0.31254	0.0781	1.30765	0.9375
	VNR	084789	0.8984	0.77164	0.3652	0.91007	0.9863
CD5	VR	0.31871	0.0156*	0.41512	0.1563	1.30252	1.0000
	VNR	0.70519	0.4648	0.84308	0.7002	1.19554	0.7646
CDC2	VR	1.83308	0.0469	2.44845	0.0156*	1.33570	0.3750
	VNR	0.95772	0.9658	1.01598	0.8984	1.06083	0.8311
CDKN2A	VR	0.75566	0.6875	1.52407	0.6875	2.01686	0.3750
	VNR	1.59954	0.1016	2.11371	0.0098*	1.32145	0.1055
CSF2	VR	0.18770	0.5781	0.28488	0.0156*	1.51773	1.0000
	VNR	0.71349	0.8984	1.08977	0.8984	1.52738	0.5771
CXCL9	VR	0.08858	0.0156*	0.31447	0.5781	3.54993	0.5781
	VNR	1.20657	0.7646	0.70477	0.8984	0.58411	0.9658
GREB1	VR	1.01129	0.9375	0.48025	0.3750	0.47489	0.0156*
	VNR	0.77097	0.9658	1.46296	0.3652	1.89755	0.1973
HSPB1	VR	1.54018	0.4688	0.74418	0.3750	0.48318	0.0156*
	VNR	1.46353	0.2783	1.11214	0.3203	0.75990	0.3203
IL1A	VR	0.42877	0.8125	1.52009	0.4688	3.54527	0.3750
	VNR	2.28078	0.1230	2.53624	0.0244*	1.11200	0.9658
IL2	VR	0.27657	0.2188	0.83277	1.0000	3.01108	0.0313*
	VNR	1.09551	0.5195	2.07814	0.2061	1.89696	0.3652
NAIP	VR	0.11808	0.0156*	0.23694	0.0781	2.00666	0.8125
	VNR	0.70448	1.0000	1.01617	1.0000	1.44243	0.3652
NFKB1	VR	0.78420	0.8125	0.99867	0.9375	1.14238	1.0000
	VNR	1.92877	0.0674	2.11918	0.0244*	1.09872	0.7646
TERT	VR	0.15595	0.3750	0.17627	0.2188	1.13030	0.2188
	VNR	1.04825	0.6377	4.84834	0.0068*	4.62519	0.1016
TFRC	VR	1.54390	0.2188	0.64171	0.4688	0.41564	0.0156*
	VNR	1.75120	0.1230	1.59652	0.1748	0.91167	0.8984
TNF	VR	0.36723	0.0313*	0.27245	0.0156*	0.74190	0.4688
	VNR	0.77058	0.6377	1.01372	0.5195	1.31554	0.3203
TP53	VR	1.32883	0.6875	0.64317	0.3750	0.48401	0.0313*
	VNR	1.60496	0.1016	1.75163	0.2061	1.09138	0.8311
VCAM1	VR	0.27805	0.0469*	0.36130	0.0781	1.29938	0.9375
	VNR	0.92072	0.8984	0.63846	0.5771	0.69344	0.4648

Differential expression of genes was determined using RNA array data and comparison between the biopsies obtained as described in the text. Statistical analyses were performed and relative fold changes (RQ) were calculated from baseline expression, * $p < 0.05$.

Abbreviations: *BIRC3* (Baculoviral IAP repeat containing 3), *CD5* (Cluster of differentiation 5), *CDC2* (Cell Division Control 2), *CDKN2A* (Cyclin-dependent kinase inhibitor 2A), *CSF2* (Colony Stimulating Factor 2), *CXCL9* (Chemokine (C-X-C motif) Ligand 9), *GREB1* (Growth Regulation by Estrogen in Breast cancer), *HSPB1* (Heat Shock Protein Beta-1), *IL1A* (Interleukin 1 Alpha), *IL2* (Interleukin 2), *NAIP* (NLR family, apoptosis inhibitory protein), *NFKB1* (Nuclear factor of Kappa Light Polypeptide Gene Enhancer in B cells), *TERT* (Telomerase reverse transcriptase), *TFRC* (Transferrin receptor), *TNF* (Tumor Necrosis Factor), *TP53* (Tumor Protein 53), *VCAM1* (Vascular Cell Adhesion Molecule 1).

with a significance level of $p < 0.05$.

Of the 192 genes assessed, 17 gene expression changes met criteria for both biological and statistical significance; 13 and 4 genes were found to be differentially expressed in VR and VNR, respectively (Table 1). Interestingly, among these differentially expressed genes (DEG), all but two genes (*IL2* and *CDC2*) in VR were down-regulated; in contrast, all four DEG in VNR were upregulated.

The expression pattern identified in this study confirms our previous findings, providing further evidence that sinecatechins mediates EGW regression through pro-apoptotic expression changes. Notably, while the anti-apoptotic gene *CDC2* was found to be down-regulated only in the present study, *BIRC1* and *BIRC3* — which encode inhibitors of apoptosis proteins and are known targets of HPV E6 — as well as *TNF* were down-regulated in VR in both studies (Yu et al., 1998; James et al., 2006). *BIRC3*'s consistent down-regulation in response to sinecatechins treatment likely marks the reversal of a viral pathomechanism known to prevent apoptosis. Similarly, *TNF* can be either pro- or anti-apoptotic but is predominantly the latter, and to this end, the blockage of *TNF* is a therapeutic strategy in the management of a variety of diseases, including plaque psoriasis and rheumatoid arthritis. This is not the first time green tea derivatives have been shown to down-regulate *TNF*; recently, epigallocatechin gallate (EGCG), which is an active constituent in sinecatechins ointment, was shown to effectively down-regulate *TNF in vitro* (Wang et al., 2014). These findings provide strong evidence for *TNF* as a key therapeutic target in the treatment of EGW.

Based on results from this and our previous study, sinecatechins ointment appears to interact with the NF-κB pathway to modulate apoptosis. The NF-κB pathway, however, functions in other biological processes, including inflammation. Another NF-κB associated change includes *NFKB1*, which encodes the p105 subunit of a host of transcription factors active in the NF-κB pathway. Interestingly, *NFKB1* was up-regulated in VNR, which is counterintuitive to the aforementioned down-regulation of NF-κB-pathway molecule *BIRC3* in VR. However, taken together with the finding that the pro-inflammatory *IL1A* is also upregulated in VNR, the up-regulation of *NFKB1* in VNR likely reflects the uncontrolled inflammation in lesions that sinecatechins was unable to suppress. It is unclear whether the inflammation would have occurred independent of sinecatechins application; regardless, inflammatory changes appear to be an important indicator of therapeutic failure. In contrast, several pro-inflammatory genes were down-regulated in VR, including *VCAM1*, which promotes inflammatory cell migration; *CD5*, which serves as a marker for T cell proliferation in the inflammatory response; *CSF-2*, which stimulates differentiation of inflammatory cells such as granulocytes and macrophages; and *CXCL9*, which encodes the chemoattractant that functions in tandem with interferon-gamma in the recruitment of macrophages (Yusuf-Makagiansar et al., 2002; Saha et al., 2010).

Although the HPV types detected in the VR' lesions are uncommonly associated with malignancy (HPV-6, -18, and -35), dysregulated epithelial cell proliferation is a cardinal pathophysiological feature of EGW. To this end, it is not surprising that several genes implicated in tumor immortalization were down-regulated and up-regulated in VR and VNR, respectively. Cancer-associated genes with decreased expression levels in VR include *GREB1* (breast cancer) and *TFRC* (leukemia and lymphoma) (Aisen, 2004; Liu et al., 2012). Interestingly, *TP53*, the gene for the well-described tumor suppressor p53, was down-regulated in VR; while the significance of this finding is unclear, this expression change could represent a return-to-normal cellular function in VR as the sinecatechins-treated lesions resolve. The down-regulation of *HSPB1*, which functions to facilitate cellular stress response, may also underlie a return-to-normal cellular response in VR. In contrast, *TERT*, which encodes the telomerase reverse transcriptase enzyme, was up-regulated in VNR by approximately 4.85×. Telomerase up-regulation is highly associated with carcinogenesis since upregulation promotes apoptosis bypass and promotes immortalization (Baird, 2010). Finally, *CDKN2A* was upregulated 2.1× in VNR. *CDKN2A* encodes $p16^{INK4A}$, which inhibits growth signals leading to G1 growth arrest and senescence, and thus, *CDKN2A* is traditionally known as a tumor suppressor gene. However, it was shown that $p16^{INK4A}$ is upregulated in HPV-mediated tumors and that this upregulation is necessary for tumor growth *in vitro*. It is hypothesized that the HPV E7 protein stimulates $p16^{INK4A}$, resulting in a molecular state in which the iconoclastic tumor suppressor $p16^{INK4A}$ actually stimulates cell proliferation (McLaughlin-Drubin et al., 2013). The up-regulation of *CDKN2A* in our study provides *in vivo* evidence for this recently described *in vitro* process, which itself represents a potential therapeutic target.

Taken together with our previous report, current results provide cursory evidence that the sinecatechins' mechanism of action likely entails some degree of modulation of inflammatory and apoptotic processes, in particular, the NF-κB-pathway. It is possible that sinecatechins also upregulate the host immune response, since the only gene to be significantly upregulated in VR is the pro-lymphocytic *IL2*; however, further studies are needed to examine the exact pattern of immune regulation. Moreover, since no DEGs were identified in VNR in the prior report, the present study provides putative insight into the expression patterns of patients who are not re-

sponsive to sinecatechins. Further examination into the mechanism of resistance is warranted, perhaps through the use of gene expression to identify patients who will not benefit from sinecatechins therapy. Overall, these studies provide valuable preliminary *in vivo* evidence on how sinecatechins may function to treat a highly infectious and debilitating disease.

FOOTNOTES

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