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Upper esophageal and pharyngeal cancers

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Abstract

The following, from the 12th OESO World Conference: Cancers of the Esophagus, includes commentaries on laryngopharyngeal reflux as a risk factor for laryngeal cancer; the role of pepsin in laryngopharyngeal neoplasia; natural fruit and vegetable compounds for the prevention and treatment of pharyngeal and esophageal cancers; and evaluation of cranberry constituents as inhibitors of esophageal adenocarcinoma utilizing *in vitro* assay and *in vivo* models.

Keywords

laryngopharyngeal reflux; laryngeal cancer; cranberry; red beetroot dye; esophageal cancer; OESO

Concise summary

Given that the laryngopharynx is more susceptible to damage by gastric refluxate compared to the esophagus and the lack of intrinsic defense mechanisms present in their esophageal counterparts (such as peristalsis, saliva, and bicarbonate production), it is plausible that chronic uncontrolled laryngopharyngeal reflux (LPR) could not only cause inflammatory, but perhaps also neoplastic, pathologies in the laryngopharynx, as it does in the esophagus. Patients with LPR have pepsin-mediated laryngeal mucosal injury, including dilation of intracellular spaces and mitochondrial damage. Pepsin is taken up by laryngeal epithelial cells through receptor-mediated endocytosis. Receptors and their ligands are typically sorted in late endosomes and the transreticular Golgi (TRG). Using immunoelectron microscopy, colocalization of pepsin with Rab-9 and TRG-46, markers of late endosomes, and the transreticular Golgi, respectively, was documented. These findings reveal an entirely novel mechanism for pepsin-induced cellular injury and may explain why many patients with reflux-attributed laryngeal injury and disease often have persistent symptoms despite maximal acid-suppression therapy and association with nonacid reflux. Thus, the activity/

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Conflicts of interest

The authors declare no conflicts of interest.

stability data and finding of pepsin uptake and intracellular pepsin reveal a role for pepsin in extraesophageal reflux, where the acidity of the refluxate may not be as clinically relevant.

Color compounds from freeze-dried black raspberries have significant anti-inflammatory and anticancer activity, and several studies have shown the ability of black raspberry anthocyanins to inhibit the growth of esophageal papillomas in an esophageal cancer model in rats. Recent work has shown that esophageal papilloma induction was reduced 45% in a rat esophageal cancer model by continuous consumption of red beetroot dye (RBD) in the animal's water through a 35-week exposure, and potent antiproliferative effects of RBD in direct treatment of pharyngeal cancer cell lines, with dose-dependent toxicity, were noted in cell growth and proliferation assays. Work is ongoing to determine the effects of RBD on cell cycle distribution and angiogenesis in nude mouse xenograft models. This product has a very high potential for direct translational application in the clinic owing to easy preparation, low cost, and ease of use.

Intraperitoneal injection of cranberry extracts increases tumor latency and inhibits growth of gastric, colon, prostate, lymphoma, and glioblastoma cancer cells, especially the cranberry proanthocyanidins (C-PACs) fraction, supporting *in vivo* efficacy given direct administration. C-PACs have potent cell death-inducing capacity against esophageal, lung, and colon cancer cells, at much lower concentrations compared to strawberry or black raspberry constituents. They significantly induce esophageal adenocarcinoma (EAC) cell death via apoptosis, autophagy, and necrosis. They also induce protein expression of cell cycle markers (p16/p21) and cause an S-phase delay and cell cycle arrest at G2/M in adenocarcinoma cells. MAPK and NF- κ B pathways are two of the major signaling cascades documented to be influenced by acid refluxate, resulting in an apoptosis-resistant phenotype. Cranberry constituents appear particularly well suited to combat reflux-induced molecular changes and subsequent EAC. The fact that C-PAC kills adenocarcinoma cells by different mechanisms based on the oxidative state of the cell is promising, affording opportunities for prevention and potentially novel therapeutic strategies.

1. Can LPR promote carcinogenesis of the laryngopharynx?

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The American Cancer Society estimated that in 2013 there would be 12,260 new cases of laryngeal squamous cell carcinoma and 3,630 deaths attributed to laryngeal carcinoma. They also estimated 13,930 new cases of pharyngeal carcinoma, with 2400 deaths in 2013.¹ Despite a decrease in the number of people who smoke in the United States, the incidence of laryngeal cancer actually appears to be rising. Unfortunately, the prognosis remains poor and the mortality rate high, with a 5-year survival rate of 40%. Tobacco and alcohol are well-known established risk factors. Other risk factors include human papilloma virus, radiation exposure, and LPR. It is well known and accepted that chronic reflux into the esophagus causes reflux esophagitis, which can, over time, result in a metaplastic change in the cells that line the lower esophagus—Barrett's esophagus (BE). This metaplasia confers an increased risk of EAC. Chronic inflammation in reflux esophagitis is known to create a

favorable environment for DNA damage, increased proliferation, and inhibition of apoptosis.² Given that the laryngopharynx is more susceptible to damage by gastric refluxate compared to the esophagus, and the lack of intrinsic defense mechanisms present in their esophageal counterparts (such as peristalsis, saliva, and bicarbonate production), it is plausible that chronic uncontrolled LPR could not only cause inflammatory, but perhaps also neoplastic, pathologies in the laryngopharynx, as it does in the esophagus.

We have previously reported that patients with LPR have pepsin-mediated laryngeal mucosal injury, including dilation of intracellular spaces and mitochondrial damage.³ The expression of laryngeal stress proteins and protective proteins, such as carbonic anhydrase (which neutralizes acid) and mucins (which compose the mucosal barrier), is depleted in LPR patients.⁴ Furthermore, proinflammatory cytokine expression profiles in LPR patients, and in hypopharyngeal cells in response to pepsin, are similar to those observed in the esophagus in response to gastroesophageal reflux disease (GERD).⁵ These studies demonstrate a role for LPR in laryngeal inflammatory disease, similar to what is known for GERD in the esophagus.

For many reasons, it is very difficult to assess the role of reflux in laryngeal cancer. First, while many clinical studies have shown a high prevalence of LPR in patients with laryngeal cancer, these studies are confounded by the fact that the majority of patients with laryngeal cancer have a significant smoking and alcohol history. Second, there is a lack of uniformity in establishing the diagnosis of LPR in the literature. And, third, epidemiological studies that have investigated an association between LPR and laryngeal cancer were somewhat skewed because there was no specific diagnostic code for LPR, the current procedural terminology code included GERD, and most GERD patients do not have proximal LPR symptoms and injury, therefore potentially diluting any associations.

Gabriel and Jones were among the first to present evidence suggesting that chronic laryngitis from LPR is associated with, and may promote, cancer of the laryngopharynx.⁶ In 2003, Night *et al.* reported a high frequency of reflux in laryngeal cancer patients who do not drink or smoke, resulting in an increased interest in the relationship between reflux and laryngeal cancer.⁷ Last year, Cekin *et al.* reported LPR in 69.8% of new patients presenting with a laryngeal lesion. When these patients were analyzed in subgroups based on whether their lesion was benign or malignant/premalignant, they found a significant relationship between LPR positivity (measured by the reflux symptom index and reflux finding score) and the presence of a malignant/premalignant lesion ($P = 0.03$).⁸ Interestingly, several clinical studies evaluating patients with prior gastrectomy suggest that the components of nonacidic reflux promote the development of laryngeal cancer.⁹ Initial investigations of LPR-attributed disease focused on the effects of gastric acid. However, injury is seen despite proton pump inhibitor (PPI) therapy, and injury and symptoms are associated with nonacidic reflux events.¹⁰

We have reported that pepsin is taken up by laryngeal epithelial cells through receptor-mediated endocytosis. Receptors and their ligands are typically sorted in late endosomes and the transreticular Golgi. Using immunoelectron microscopy, colocalization of pepsin with Rab-9 and TRG-46, markers of late endosomes and the transreticular Golgi, respectively,

was documented. The pH of the TRG and/or endosomes—a pH of approximately 5—is well documented in the literature. Pepsin has approximately 40% of its maximal activity at this pH. Thus, when inactive pepsin is taken up by airway epithelial cells, it could potentially be reactivated in these intracellular compartments of lower pH and subsequently cause intracellular damage.

These findings reveal an entirely novel mechanism for pepsin-induced cellular injury and may explain why many patients with reflux-attributed laryngeal injury and disease often have persistent symptoms, despite maximal acid-suppression therapy, that are associated with nonacid reflux. Thus, while pepsin is known to play a role in GERD at low pH owing to its proteolytic activity, the activity/stability data and finding of pepsin uptake and intracellular pepsin reveal a role for pepsin in extraesophageal reflux, where the acidity of the refluxate may not be as clinically relevant.

We have recently demonstrated that pepsin is detected in the larynges of cancer patients but absent in patients without clinical signs of reflux or inflammatory and neoplastic disease. Our *in vitro* studies demonstrate that pepsin induces a dose- and time-dependent promotion of proliferation in both normal laryngeal and transformed hypopharyngeal epithelial cultures, and thus may play a role in carcinogenesis of the laryngopharynx by causing aberrant cell growth. This induction of proliferation is associated with gene and microRNA expression changes that are consistent with promotion of neoplasia.¹¹ Chronic pepsin exposure also caused resistance to apoptosis, another hallmark of a tumor promoter (unpublished data). Taken together with (1) the *in vivo* hamster study demonstrating an increase in tumor volume in animals exposed to active pepsin¹² and (2) an increase in colony-forming ability in nonacid pepsin-treated cells relative to controls, these data reveal that chronic pepsin exposure will promote tumorigenesis, highlighting a role for LPR of pepsin in carcinogenesis of the laryngopharynx.

In summary, LPR (pathology and the number of episodes) is more prevalent in patients with laryngeal cancer (as is the presence of pepsin), but larger clinical studies are needed to demonstrate a causal relationship because of the confounding effects of tobacco and alcohol. The lack of both a good diagnostic test for LPR and an independent diagnostic code to separate GERD from LPR complicates such studies. *In vitro* and *in vivo* studies strongly suggest a role for LPR in carcinogenesis of the laryngopharynx, but further cell and molecular biology studies are needed to elucidate the exact role and mechanism. It is important to note that receptor-mediated uptake of nonacid pepsin, as in nonacid LPR, and the subsequent inflammatory and neoplastic changes that occur, will not be prevented by PPIs, since the pepsin appears to become reactivated inside intracellular compartments of lower pH, such as the TRG and late endosomes.

2. RBD for the treatment of pharyngeal and upper esophageal cancers

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Head and neck cancer is now the fifth most common new cancer diagnosis in the United States, with over 40,000 new cases of oral cavity and pharyngeal cancer diagnosed in 2012.¹ The most common head and neck malignancy is squamous cell carcinoma of the head and neck. Overall survival rates for patients with pharyngeal cancer have not changed markedly over the last three decades, despite great progress in oncologic therapies. Many investigators have evaluated exogenous chemical compounds for the ability to prevent the development of primary and secondary head and neck malignancies, a concept known as chemoprevention. Chemopreventive agents, including retinoic acid¹³ and nonsteroidal anti-inflammatory drugs,^{14,15} have been investigated in both preclinical and clinical oropharyngeal cancer models. To date, these studies have failed to show clinical utility, and further studies are needed to identify novel agents for use in pharyngoesophageal cancer chemoprevention.

Studies of natural plant compounds as inhibitors of cancer development and progression have increased markedly in the last several years. Color compounds from freeze-dried black raspberries have significant anti-inflammatory and anticancer activity, and recent studies have shown the ability of black raspberry anthocyanins to inhibit the growth of esophageal papillomas in an esophageal cancer model in rats.¹⁶ Direct application of anthocyanin compounds to oral precancerous lesions can specifically promote tissue differentiation in the oral cavity and promote favorable changes in gene expression.^{17,18} Following these studies, beetroot extracts have also been evaluated for activity against human cancers. RBD, sold in the food industry as red dye E162 (CHR Hansen, Milwaukee, Wisconsin), is known to contain high levels of the red dye compounds betacyanin and betalain. These appear to have similar activity against some human tumors through inhibition of free radical generation and inhibition of human tumor progression, in preclinical models.¹⁹ Recent work has shown that continuous consumption of RBD in water over 35 weeks reduced esophageal papilloma induction by 45% in a rat esophageal cancer model.²⁰ Subsequent studies have also shown similar chemopreventive effects in lung tumor models.²¹ No significant toxicity was seen in the animals from these treatment protocols. The ability of RBD to inhibit oral or pharyngeal cancer growth has not yet been investigated; however, initial work from our laboratory has shown potent antiproliferative effects of RBD in direct treatment of pharyngeal cancer cell lines, with dose-dependent toxicity noted in cell growth and proliferation assays (Fig. 1), and work is ongoing to determine the effects of RBD on cell cycle distribution and angiogenesis in nude mouse xenograft models.

Oral and pharyngeal cancer is an excellent model for the study of chemopreventive agents, such as RBD, owing to the ease of examining precancerous and cancerous lesions in the clinic. RBD is also very soluble in water and has excellent bioavailability, making application of the betacyanin and betalain compounds in RBD feasible through both local administration (mouth rinses) and systemic absorption in cancer patients. We believe that this product has a high potential for direct translational application in the clinic due to easy preparation, low cost, and ease of use.

3. Cranberries hold cancer inhibitory potential for targeting EAC

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Cranberries are rich in bioactive constituents that reportedly influence a variety of health outcomes, including improved immune function, decreased urinary tract infections, modulation of inflammatory processes, reduced cardiovascular disease, and more recently, cancer inhibition.^{22,23} Over 150 individual phytochemicals have been identified from cranberries, as extensively reviewed by Pappas and Schaich.²² Flavonoids composed of proanthocyanidins (PAC), anthocyanins, flavonols, and flavan-3-ols dominate the composition of cranberries and are postulated to contribute to cranberry-induced favorable biologic effects. Extensive preclinical and clinical evaluations regarding the urinary tract health benefits of cranberries has determined that the unique A-type linkages predominantly found in C-PACs prevent adhesion of P-fimbriated uropathogenic *Escherichia coli*.²⁴ However, there is surprisingly little mechanistic information regarding the ability of cranberry constituents to inhibit cancers, particularly *in vivo*.

Cranberry research focused on the inhibition of cancer or precancerous lesions is summarized in Table 1, with Table 2 outlining specific cancer-linked pathways altered by cranberries. Neto²³ reviewed cancer-linked cranberry research conducted through early 2011 (Table 1). The vast majority of research assessing cranberries as cancer inhibitors has been conducted *in vitro*, utilizing a number of cranberry extracts and diverse human cancer cell lines (Table 1),^{22,23,25–29} including our research focused on EAC inhibition. Briefly, intraperitoneal injection of cranberry extracts, especially the C-PAC fraction, increases tumor latency and inhibits growth of gastric, colon, prostate, lymphoma, and glioblastoma cancer cells,^{22,23,27} supporting *in vivo* efficacy of C-PACs when administered directly. Only two *in vivo* studies using chemical carcinogen induction and oral delivery of cranberries have been reported. A study conducted by Boateng *et al.* used azoxymethane induction of aberrant crypt foci in F344 rats and reported that 20% cranberry juice significantly reduces aberrant crypt foci in the proximal and distal colon and increases liver glutathione-S-transferase levels (reviewed in Ref. 22). In another study by Prasain *et al.*, a cranberry juice concentrate administered by gavage significantly reduced nitrosamine-induced bladder tumor weight and cancerous lesion formation in the bladder of F344 rats, but the authors lacked a mechanistic explanation for the effects (reviewed in Ref. 23).

Considering that increased consumption of plant-based diets, rich in fruits, vegetables, antioxidants, and fiber, are generally associated with a reduction in the risk for EAC, whereas those of animal origin are associated with risk escalation,³⁰ our lab began testing naturally derived extracts as EAC inhibitors. We found that C-PACs had potent cell death-inducing capacity against esophageal, lung, and colon cancer cells, at much lower concentrations compared to strawberry or black raspberry constituents. Thus, we embarked on a series of pre-clinical studies to evaluate mechanisms associated with the cancer inhibitory potential of C-PAC, using a panel of human EAC cell lines (JHAD1, OE33, OE19) and, in parallel, assessing inhibition of OE19 tumor xenografts in athymic NU/NU mice.^{25,26} Our results show that C-PAC significantly induces EAC cell death via apoptosis, autophagy, and necrosis. Cell death induction by C-PAC was caspase independent but linked to proapoptotic protein activation (BAX, BAK1, cytochrome C, PARP), cell line-selective MAPK (P-p38/P-ERK/P-JNK) modulation, and cell line-specific reactive oxygen

species (ROS) effects. C-PAC maximally induces ROS levels in JHAD1 and OE33 cells, resulting in cell death via apoptosis and autophagy; yet C-PAC modestly increases ROS levels in OE19 cells, resulting in necrotic cell death.²⁶

C-PAC also induces protein expression of cell cycle markers (p16/p21) and causes an S-phase delay and cell cycle arrest at G2/M in EAC cells. C-PAC differentially affected expression of additional cancer-linked proteins, including P-ERK1/2, BCLxL, MMP2, TRF-1, and P-4EBP1 in JHAD1 versus OE19 cells, potentially linking it to differing constitutive oxidative states, variable apoptosis resistance, and, in turn, selective activation of cell death pathways. Next, we attempted to establish EAC xenografts in NU/NU mice; however, only OE19 cells readily developed tumor xenografts. Gavage administration of C-PAC significantly inhibits the growth of OE19 xenografts via modulation of apoptotic, cell cycle, and MAPK signaling, supporting *in vivo* efficacy and paralleling *in vitro* results.

Table 2 summarizes cancer-linked signaling networks modulated by cranberry constituents on the basis of our research and the published literature.^{22,23,25,26,28} In addition, we evaluated C-PAC modulation of microRNAs. C-PAC treatment of JHESoAD1, OE33, and OE19 cells modulated five microRNAs in common, resulting in 26 validated gene targets and identification of various cancer signaling pathways.²⁵ Multiple software tools were employed to interrogate the results, with common pathways identified as altered by C-PAC in EAC cells, including apoptosis, cell cycle, DNA damage, P53 signaling, cytokine–cytokine receptor interaction, p38 MAPK, immune response, focal adhesion, and angiogenesis, as summarized in Table 2.²⁵

Positive attributes of cranberries for cancer prevention include that they are naturally occurring, widely cultivated, readily available, structurally characterized to the extent that modern technologies permit, and elicit biological effects linked to positive health outcomes when administered orally at nontoxic concentrations in humans (up to 75 mg/day)³¹ and in rodents (33 g/kg; reviewed in Ref. 23). Optimally, agents targeting EAC or BE should counteract reflux-induced alterations in cancer-related signaling networks. Pathways that support a sustained growth advantage in damaged esophageal cells lead to acid resistance, alterations in susceptibility to apoptosis, activation of pro-survival mechanisms, and ultimately in neoplastic progression of Barrett's to EAC. MAPK and NF-κB pathways are two of the major signaling cascades documented to be influenced by acid refluxate, resulting in an apoptosis-resistant phenotype.³¹ Acid has been linked to activation of p38/ERK/ MAPKs and more recently to increased cytokines and NF-κB–linked inflammatory molecules. On the basis of our experimental findings, cranberry constituents appear particularly well suited to combat reflux-induced molecular changes and subsequent EAC. The fact that C-PAC kills EAC cells by different mechanisms based on the oxidative state of the cell is promising, affording opportunities for prevention and potentially novel therapeutic strategies. To conclude, the preclinical results of C-PAC inhibiting EAC are encouraging; however, additional *in vivo* and early-phase clinical research focused on cranberry characterization, structure–activity linkages, bioavailability and metabolism, microflora profile effects, mode and matrix of delivery, and specific mechanisms of inhibition in heterogeneous esophageal tissues and at-risk patient populations, is needed.

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Figure 1.

Red beetroot dye inhibits proliferation in SCCHN. Red beetroot dye was solubilized in water and applied in standard media to oral cavity (scc1) and pharyngeal (17b) cancer cell lines for 48 h. Cells were treated with trypan blue and counted on a hemacytometer. Error bars show standard deviations, * $P < 0.05$ compared to untreated control.

Table 1

Summary of preclinical evaluations of cranberries or cranberry constituents as cancer inhibitors

Target organ (Reference)	<i>In vitro</i> models	<i>In vivo</i> models; cranberry constituent and mode of delivery; results
Oral cavity (reviewed in Refs. 22 and 23)	CAL27, SCC25, KB	
Esophagus (Ref. 25 and 26)	JHEsoAD1, OE19, OE33	OE19 xenografts in athymic NU/NU mice; proanthocyanidin-rich cranberry extract via oral gavage; decreased tumor size by 67% and affected multiple cancer signaling pathways
Stomach (Ref. 27)	SGC-7901	SGC-7901 xenografts in Balb/c mice; SGC-7901 cells were pretreated with cranberry extract prior to xenograft implant; increased tumor latency and reduced tumor size in a dose-dependent manner.
Colon (reviewed in Refs. 22 and 23)	HT-29, HCT116, SW460, SW620	AOM-induced ACF in F344 rats; 20% cranberry juice; reduced ACF formation in the proximal and distal colon and increased levels of liver glutathione-S-transferase. HT29 xenografts in Balb/c mice; flavonoid-rich fraction and proanthocyanidin-rich fraction via IP injection; inhibition of explant growth by the PAC fraction
Lung (reviewed in Refs. 22, 23, & 28)	NCI-H460	
Breast (reviewed in Refs. 22 and 23)	MDA-MB-435, MCF-7	
Cervix (reviewed in Ref. 22)	ME180	
Ovary (reviewed in Refs. 22 and 29)	SKOV-3	
Prostate (reviewed in Ref. 23)	DU-145, PC3, RWPE-1, RWPE-2, 22Rv1	DU-145 xenografts in Balb/c mice; flavonoid-rich cranberry fraction and proanthocyanidin-rich fraction via IP injection; inhibition of explant growth by the PAC fraction
Bladder (reviewed in Ref. 23)		Nitrosamine-induced F344 rat; cranberry juice concentrate via gavage; 31% reduction in bladder tumor weight and 38% reduction in cancerous lesion formation
Leukemia (reviewed in Ref. 23)	K562	Syngeneic mice; cranberry juice extract via IP injection; inhibition of Rev-2-T-6 tumor growth
Lymphoma (reviewed in Ref. 23)	Rev-2-T-6	
Glioblastoma (reviewed in Ref. 23)	U87	U87 xenografts in Balb/c mice; flavonoid-rich cranberry fraction and proanthocyanidin-rich fraction via IP injection; both fractions slowed tumor growth.
Neuroblastoma (Ref. 29)	SH-Sy5Y, IMR-32, SMS-KCNR	

Table 2

Cancer signaling networks altered by cranberry treatment

Cell cycle via G2/M and G1/S arrest
Cell death via apoptosis, autophagy, and necrosis
MAPK
PI3K/AKT/mTOR
Inflammation
Cytokine–cytokine
Oxidative stress
Angiogenesis
Invasion and metastasis
Immune response
P53
DNA damage
Cell adhesion
Differentiation

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