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Molecular Mechanisms of Ethanol-associated Oro-esophageal Squamous Cell Carcinoma

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Abstract

Alcohol drinking is a major etiological factor of oro-esophageal squamous cell carcinoma (OESCC). Both local and systemic effects of ethanol may promote carcinogenesis, especially among chronic alcoholics. However, molecular mechanisms of ethanol-associated OESCC are still not well understood. In this review, we summarize current understandings and propose three mechanisms of ethanol-associated OESCC: (1) Disturbance of systemic metabolism of nutrients: during ethanol metabolism in the liver, systemic metabolism of retinoids, zinc, iron and methyl groups is altered. These nutrients are known to be associated with the development of OESCC. (2) Disturbance of redox metabolism in squamous epithelial cells: when ethanol is metabolized in oro-esophageal squamous epithelial cells, reactive oxygen species are generated and produce oxidative damage. Meanwhile, ethanol may also disturb fatty-acid metabolism in these cells. (3) Disturbance of signaling pathways in squamous epithelial cells: due to its physico-chemical properties, ethanol changes cell membrane fluidity and shape, and may thus impact multiple signaling pathways. Advanced molecular techniques in genomics, epigenomics, metabolomics and microbiomics will help us elucidate how ethanol promotes OESCC.

Keywords

ethanol; oro-esophageal squamous cell carcinoma; molecular mechanisms

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1. Overview

Cancers of the upper aerodigestive tract (oral cavity, pharynx, larynx, and esophagus) represent a major public health problem worldwide, making up nearly 4.4% of all malignancies in the United States alone. Approximately 73,240 new cases were diagnosed in 2014, with an estimated mortality of 27,450 [1]. Among these cancers, the incidence rates of oro-esophageal cancers have been increasing in the developed countries, especially among young males [2,3]. The most common malignancy in the oro-esophagus, oro-esophageal squamous cell carcinoma (OESCC) develops from precancerous lesions, and histopathologically follows a step-wise pattern of hyperplasia, dysplasia and squamous cell carcinoma (SCC) [4]. In the United States, the five-year survival rate has not improved significantly despite advances in radiotherapy and chemotherapy [1]. Survivors are usually left with severe functional compromise [5]. Moreover, 2% of esophageal cancer and 11% of head and neck cancer patients develop a second cancer due to field cancerization [6,7].

Many epidemiological studies have consistently shown that alcohol drinking is an etiological factor of human OESCC. OESCC has a stronger association with alcohol drinking than do cancers of other organ sites. According to a meta-analysis, strong direct trends in risk were observed for cancers of the oral cavity and pharynx (Relative Risk (RR) =6.0 for 100 g/day of ethanol), the esophagus (RR=4.2) and the larynx (RR=3.9) [8]. Risk and mortality of OESCC were associated with alcohol drinking in a dose-dependent manner [8–10]. Meanwhile, the odds ratio of oral cancer patients with dysplasia increased with alcohol consumption [11]. Genetic polymorphisms of ethanol-metabolizing genes, such as acetaldehyde dehydrogenase (ALDH) and alcohol dehydrogenases (ADH), are associated with OESCC [12–14]. Several studies have identified ALDH2*1/2 heterozygotes as a high-risk group for OESCC [15–19], and ADH genotypes not including ADH3 were associated with OESCC as well [13,20,21]. Tobacco use and alcohol drinking have synergistic effects on carcinogenesis; combined use explained more than 61% of OESCC [22,23].

After ingestion, ethanol is rapidly absorbed through the stomach and small intestine into the blood stream, and metabolized mainly in the liver before elimination. Pulmonary and urinary elimination is minimal. In the liver, ethanol is oxidized to acetaldehyde by ADH. A small amount is oxidized by cytochrome P450 2E1 (CYP2E1) and catalase. Acetaldehyde is released from the liver and metabolized into acetate by ALDH. Finally, acetate is oxidized to produce carbon dioxide, fatty acids and water. Previous studies have shown that CYP2E1 also catalyzes conversion of ethanol to acetaldehyde, and acetaldehyde to acetate [24,25].

Both ethanol and acetaldehyde may enter oro-esophageal epithelial cells through local permeation or systemic circulation. Ethanol concentration in the saliva is equal to concentration in the blood. In the saliva, ethanol is oxidized by microbes to acetaldehyde. Since further metabolism of acetaldehyde to acetate by oral bacteria is limited, acetaldehyde tends to accumulate in the saliva [26–28]. Antiseptic can significantly reduce salivary acetaldehyde concentration after alcohol drinking for this reason [27]. Tobacco changes the oral bacterial flora rapidly from Gram-negative to Gram-positive bacteria, and leads to a high concentration of acetaldehyde in saliva [29]. In agreement with these observations, alcoholics with oropharyngeal cancer had a high concentration of salivary acetaldehyde as a

result of alcohol drinking, tobacco smoking and poor oral hygiene [30]. However, due to the short duration of contact and limited permeation into the epithelium, topical effects of ethanol and acetaldehyde on squamous epithelial cells are potentially weak. Presumably only superficial cells in the epithelium may be impacted by this mechanism *in vivo* [31].

Local and systemic effects of ethanol may influence carcinogenesis, especially among chronic alcoholics. However, the molecular mechanisms for ethanol-associated OESCC are still not well understood. Certain mechanisms of ethanol-associated cancer are supported by experimental studies of OESCC, but the majority of hypotheses are purely speculative or extrapolated from studies on cancers of other organ sites. Proposed mechanisms include: (1) Enhanced cell proliferation and altered expression of cytokeratin suggesting inhibition of squamous cell differentiation [32]; (2) Enhanced penetration of carcinogens into the squamous epithelium [31]; (3) Impaired antioxidant defense and enhanced production of reactive oxygen species (ROS) in the squamous epithelium [26]; (4) Interference with DNA repair machinery and DNA synthesis [33]; (5) Disturbed systemic metabolism of nutrients [26]; (6) Impaired immune function [34]; (7) Induced chronic inflammation and enhanced angiogenesis [35].

On one hand, further experimental studies are needed to examine these mechanisms in ethanol-associated OESCC. On the other hand, these mechanisms have not been systematized to provide an overview of how ethanol promotes OESCC. In this review, we summarize current data and propose three major mechanisms of ethanol-associated OESCC: (1) Disturbance of systemic metabolism of nutrients; (2) Disturbance of metabolism in squamous epithelial cells; (3) Disturbance of signaling pathways in squamous epithelial cells.

2. Disturbance of systemic metabolism of nutrients

2.1. Inhibition of retinol metabolism

When metabolized, ethanol impairs retinoid metabolism by inhibiting retinol metabolism to retinoic acid via competing with retinol for ADH and ALDH active sites, and by accelerating catabolism of vitamin A through induction of CYP2E1 [36]. A recent study showed that acetaldehyde inhibited formation of retinoic acid from retinal in rat esophagus *ex vivo* [37]. Lecithin:retinol acyltransferase which regulates retinol metabolism by esterifying retinol, is down-regulated in human head and neck SCC cells. In a study using knockout mice, partial retinol deficiency during carcinogen treatment promoted cell proliferation and carcinogenesis in tongue epithelium [38]. Retinoic acid is known to exert profound effects on cellular growth, differentiation, and cancer development in the oroesophageal epithelium through its interaction with receptors [36,39]. These studies suggest that ethanol promotes OESCC through inhibition of retinoic acid signaling. In fact, a retinoid X receptor agonist and a retinoic acid receptor γ selective agonist inhibited 4-nitroquinoline 1-oxide (4NQO)-induced oral carcinogenesis in mice [40].

2.2. Zinc deficiency

Alcohol abuse has long been associated with zinc deficiency [41]. Ethanol treatment down-regulates the expression of zinc transporters 1 and 4, as well as the zinc storage protein

metallothionein 1 in alveolar macrophages, disrupting zinc bioavailability [42]. ADH is a zinc metalloenzyme, and removal of zinc from ADH leads to a complete loss of its catalytic activity [43]. While zinc supplementation prevents alcoholic liver injury through attenuation of oxidative stress [43], zinc depletion is known to enhance oro-esophageal carcinogenesis in rats and mice [44,45]. Mechanistically, zinc deficiency causes extensive alterations in gene expression in mouse and rat esophageal epithelium [46–48]. In particular, a group of cancer-related pro-inflammatory genes were up-regulated (CXC and CC chemokines, chemokine receptors, cytokines and cyclooxygenase 2, S100A8/A9, and nuclear factor κ B (NF κ B)), suggesting that multiple inflammatory pathways participate in zinc deficiency-related OESCC. Consistent with this observation, zinc supplementation caused a shift to a less proliferative cancer phenotype by normalizing the inflammatory gene signature, inhibiting cell proliferation, and stimulating apoptosis [49,50].

2.3. Iron overload

Alcohol drinking has been shown to cause iron overload in the liver [51]. Ethanol increases total iron content via overexpression of genes involved in iron transport (divalent metal transporter 1, transferrin receptor 1, ferroportin, ceruloplasmin) and iron storage (L-ferritin) [52]. We have shown that iron accumulation in the esophagus promoted inflammation-associated carcinogenesis [53]. Mechanistically, iron overload may initiate and promote carcinogenesis through oxidative damage [53] and modification of the immune reaction [54]. It is expected that oxidative stress affects carcinogenesis through redox signaling pathways inside cells [55,56].

2.4. Increased requirements for methyl groups

Chronic alcoholism increases the requirements for methyl groups and causes dietary methyl group deficiency [26]. Deficiency of S-adenosylmethionine, folate and betaine, primarily due to low intake and destruction by acetaldehyde, is common in alcoholics. Inhibition of methyl group transfer regulates expression of genes involved in carcinogenesis [57,58]. DNA hypomethylation of oncogenes (e.g., c-Ha-ras, c-Ki-ras and c-fos) is associated with an increased incidence of liver cancer in rats [59,60]. These data suggest that ethanol may contribute to OESCC through aberrant gene methylation [61,62]. In addition, aberrant gene methylation may impact signaling pathways through critical pathway genes, such as Notch4 of the Notch signaling pathway [63], PTEN of the phosphoinositide 3-kinase (PI3K)/Akt pathway [64], and Wnt inhibitory factor 1 (WIF1) of the Wnt signaling pathway [65]. Recent studies have also showed that aberrant methylation of histones and non-histone proteins also modulates multiple signaling pathways [66].

3. Disturbance of redox metabolism in squamous epithelial cells

After ethanol and acetaldehyde get into epithelial cells through systemic circulation, they undergo intracellular metabolism [67]. It should be noted that ethanol metabolism in the oro-esophageal epithelial cells is different from that in the liver due to different enzyme profiles. Oral epithelium expresses χ and σ type ADH with high K_m values (ADH3 and ADH4) [68], and a negligible amount of ALDH. CYP2E1 is not normally expressed in oral epithelium, but is inducible by chronic ethanol treatment [69–71]. As a result, oro-esophageal epithelial

cells tend to accumulate acetaldehyde. This may partially explain why the oro-esophagus is more susceptible to ethanol-associated carcinogenesis when compared to other organ sites.

When ethanol is metabolized, ROS are produced in cells. ROS produced by the mitochondrial electron transport chain, CYP2E1, and cytosolic enzymes [72], result in oxidative damage of DNA, proteins and lipids. Chronic alcohol drinking promotes esophageal tumorigenesis which is associated with ROS production; administration of an antioxidant (α -tocopherol) attenuates this effect [73].

It has been well established that ethanol and its toxic metabolite, acetaldehyde, form DNA adducts and thus cause DNA mutations [74]. Acetaldehyde reacts directly with the exocyclic amino group of deoxyguanosine to form stable DNA adducts [75]. As the most abundant DNA adduct, N²-ethylidene-2'-deoxyguanosine (N²-EtidG), impairs the DNA repair system and apoptosis [26]. The level of N²-EtidG is increased in oro-esophageal epithelial cells after alcohol drinking in mice [76] and humans [77]. Moreover, the N²-EtidG level is dependent on ALDH2 and ADH genotypes. In animals, lack of ALDH2 leads to an increased level of N²-EtidG in the mouse upper aerodigestive tract after ethanol treatment [76]. In humans, the blood level of N²-EtidG was significantly higher in patients with a combination of the ADH1B*2 and ALDH2*2 alleles [78].

Excessive ethanol may also lead to generation of protein adducts with the aldehydic final products of lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (HNE) [79,80]. As the most abundant adduct species, HNE levels in mouse liver are dependent on both the dose and the duration of ethanol feeding [81]. Similarly, the amount of MDA is found to be significantly increased in the heart of rats treated with ethanol [82]. In oral leukoplakia and cancer, more positive staining for MDA and HNE adducts was observed in the dysplastic or malignant epithelial cells than in normal cells, and this staining was significantly correlated with acetaldehyde exposure [80]. HNE reacts with DNA bases such as deoxyadenosine and deoxycytidine to form highly mutagenic DNA adducts [83]. Furthermore, ethanol can induce hyperacetylation of proteins such as histone H3, p53 and SREBP-1c, perturbing hepatic function [84].

Cell membranes are sensitive to oxidative damage. The methylene groups between two double bonds of polyunsaturated fatty acid in cell membranes make these lipids highly sensitive to oxidation [79]. ROS-induced lipid peroxidation of cell membranes has been implicated in malignant transformation [79]. For the sake of self-protection or adaptation, cells respond to ethanol and ROS through at least two mechanisms. In response to oxidative stress, nuclear factor erythroid 2-like factor (Nrf2) released from Kelch-like ECH associating protein 1 (Keap1) translocates into the nucleus and induces transcription of target genes [85,86]. Nrf2 signaling plays an essential role in the protection of cells against oxidative damage and carcinogenesis [87,88]. Ethanol suppresses Nrf2 expression in alveolar epithelial cells and an Nrf2 activator (sulforaphane) blocks the effects of ethanol [89]. Activation of Nrf2 prevents ethanol-induced oxidative stress, lipid accumulation and accelerated acetaldehyde metabolism [85,90].

Cells may also respond to ethanol through modification of intracellular fatty acid metabolism. In the liver, heavy doses ethanol increase intracellular concentration of free fatty acids and block fatty acid oxidation through inhibition of peroxisome proliferator-activated receptor α [91–94]. It is interesting that in yeast cells, endospore effective 1 and elongation enzyme 1 are known to be associated with ethanol resistance [95,96]. Functional mammalian homologues of these genes, EH-domain containing genes (Ehd1, Ehd2, Ehd3, Ehd4) and very long chain fatty acid elongase (Elov13, Elov16) are associated with lipid metabolism and membrane functions. Knockdown of Ehd1 reduced cellular cholesterol and triglyceride, and disrupted free cholesterol distribution in the cell [97,98]. Elov16 deficient mice became obese and developed hepatosteatosis when fed a high-fat diet [99,100]. Inhibition of Elov13 reduced intracellular triglycerides significantly [101]. Both Elov16 and Elov13 were activated by peroxisome proliferator-activated receptor γ during adipogenesis [102].

4. Disturbance of signaling pathways in squamous epithelial cells

As a short-chain alcohol, ethanol diffuses in squamous epithelial cells. In this process, ethanol molecules are expected to accumulate near the interface region between lipids and the surrounding water. Ethanol is able to form hydrogen bonds with lipids in the bilayer, significantly change membrane fluidity and deformability, and leave the cell membrane unstable. Interestingly, ethanol has a biphasic effect on bilayer deformability as it first increases and then decreases deformability [103]. Ethanol reduces the membrane's surface tension and lateral pressure, which affects the conformations of membrane proteins [104], and disrupts protein-lipid interactions [105]. On the other hand, ethanol can activate a G-protein-gated inwardly-rectifying potassium channel via a defined alcohol pocket and changes in affinity for membrane phospholipid signaling [106]. These small changes in cell membranes can strongly affect the function of intrinsic membrane proteins. Therefore, it is conceivable that ethanol may act on multiple signaling pathways as shown below.

4.1. Toll-like receptor 4 (TLR4) signaling

Treatment with lipopolysaccharide, a TLR4 ligand, results in recruitment of CD14 and TLR4 into the lipid rafts, and thus triggers downstream TLR signaling events in cells. When cells are co-treated with ethanol, reorganization of the actin cytoskeleton is disturbed, impacting TLR4 and CD14 clustering and co-localization, and TLR4 signaling is suppressed [107]. However, TLR4 was overexpressed in OESCC *in vivo* and *in vitro* [108–110]. Lipopolysaccharide activation of TLR4 on cancer cells enhanced cell proliferation and inflammation through the PI3K/Akt and NF κ B pathways [111]. In an animal study using mice lacking the receptor for advanced glycation end product, TLR4 was found to mediate S100A8/A9-induced pro-inflammatory reaction [112]. TLR4 may also contribute to immune escape and chemoresistance [108]. TLR4 in host cells was involved in the regulation of tumor-related cachexia and tumor control [113]. Interestingly, a functional study showed defects in TLR4-mediated signaling in peripheral blood lymphocytes from oral cancer patients [114]. These data suggest that TLR4 may play different roles in epithelial cells and immune cells. It is still unknown how ethanol may promote OESCC through TLR4 signaling.

4.2. Notch signaling

Notch signaling is mediated through ligands binding to Notch receptors. The receptors are then cleaved to allow Notch intracellular domain (NICD) release from the membrane and entry into the nucleus to form a transcriptional complex with recombining binding protein for immunoglobulin kappa j (RBPJ). NICD displaces the repressive cofactors bound to RBPJ and recruits a transcriptional activator complex, which initiates transcription of Notch downstream target genes [115]. Notch is known to regulate squamous differentiation in the skin [116] and esophagus [117,118], particularly during commitment of keratinocytes to terminal differentiation via a Hes1-dependent mechanism [119,120]. In the esophagus, loss of Notch signaling perturbs esophageal squamous differentiation [117]. In the oral cavity, Notch1 is highly activated in oral epithelium differentiation, and disrupted Notch1 interferes with normal palate development [121]. Exome sequencing has shown in multiple studies that loss-of-function Notch mutations are frequently seen in OESCC [122–127].

The effects of ethanol on Notch signaling vary in different organs. Notch signaling is inhibited by ethanol in the pancreas and smooth muscle cells [128,129], but activated in endothelial cells and fetal cerebral cortex [130,131]. In the oro-esophagus, we found that alcohol drinking promoted epithelial cell proliferation and suppressed squamous differentiation in mouse forestomach, particularly after long-term treatment. Meanwhile, Notch signaling was down-regulated by ethanol treatment (unpublished data). These data suggest Notch inhibition as a potential mechanism of suppression of squamous differentiation due to alcohol drinking. Using genetically modified mice and chemical modulators of Notch signaling, we are testing Notch inhibition as a novel molecular mechanism of ethanol-associated OESCC.

4.3. Sonic hedgehog (Shh) signaling

Shh signaling is one of the most well-known developmental signaling pathways. In the ligand-producing cells, Shh-N is packaged into lipid raft domains and then shipped extracellularly to the receiving cells. Shh signaling is activated when Shh-N binds to Patched which releases its inhibition on Smoothened, leading to activation of Gli transcription factors to regulate gene expression [132,133]. In fetal alcohol syndrome models, Shh signaling is suppressed by ethanol. Shh deficiency makes animals more susceptible to fetal alcohol phenotypes. Supplementation of Shh mRNA [134–136] or cholesterol rescues these phenotypes [137]. *In vitro* studies find that ethanol perturbs proper association of active Shh ligand with lipid rafts destined for the membrane [132,133]. Furthermore, ethanol exposure leads to a decrease in the amount of membrane-associated cholesterol, which is vital for proper Shh signaling [137]. Contrary to this observation, ethanol activates Shh signaling, and thus promotes carcinogenesis in the liver [138,139]. In OESCC, Shh signaling is aberrantly activated [140–144], suggesting that ethanol may activate Shh signaling in the oro-esophagus.

4.4. Wnt signaling

Wnt signaling plays a crucial role in the regulation of cell proliferation, survival and migration [145]. Binding of Wnt to its receptor (frizzled) and co-receptors (low-density lipoprotein receptor-related protein 5 or 6) transduces a signal across the plasma membrane

and activates Wnt signaling [145]. β -catenin plays an important role in the epithelial-mesenchymal transition that characterizes tissue regeneration and cancer proliferation [146]. It is interesting that ethanol suppresses Wnt signaling in human neural stem cells [147], but activates it in the fetal cerebral cortex of humans and mice [130]. In diethylnitrosamine-induced hepatocarcinogenesis, ethanol promotes Wnt signaling in mouse liver [148]. In OESCC, higher expression of nuclear β -catenin is significantly associated with poor histological differentiation and clinical outcome [149]. Total β -catenin level in the tongue increases after treatment with a carcinogen and ethanol [150]. In esophageal SCC, overexpression of Wnt2 is found in tumor-associated fibroblasts and is correlated with poor survival [145]. These data suggest that ethanol may activate Wnt signaling in the oro-esophagus.

4.5. Transforming growth factor β (TGF β) signaling

TGF β signaling is a key regulator of epithelial cell proliferation, immune function and angiogenesis [151]. In the canonical pathway, TGF β ligands bind to the transmembrane receptor (type 2 TGF β receptor) that recruits type 1 TGF β receptor, resulting in Smad-mediated signaling. TGF β also signals through non-Smad pathways [152,153]. TGF β signaling is inhibited by ethanol in rat cerebral cortex [154]. However, TGF β is activated by ethanol in mouse liver and rat alveolar macrophage [155,156]. Meanwhile, exposure to ethanol increases fibroblast contraction, which is reduced by TGF β inhibition [157]. In head and neck SCC, loss of type 2 TGF β receptor or Smad4 not only abrogates TGF β -mediated tumor suppression, but also causes a compensatory increase in TGF β ligand expression that promotes inflammation and angiogenesis [151].

4.6. NF κ B signaling

NF κ B signaling is involved in many cellular processes, including inflammation and tumorigenesis. Once activated, the regulatory subunit phosphorylates specific Ser residues, leading to protein ubiquitination, subsequent proteasome processing and NF κ B releasing. Transcription factors accumulate in the nucleus to activate the target genes [86]. Ethanol activates NF κ B signaling and up-regulates expression of inflammatory mediators, and thus induces hepatotoxicity [158–160]. In the oro-esophagus, NF κ B alleles are associated with oral carcinogenesis [161]. It is still not known whether ethanol may regulate NF κ B signaling in oro-esophageal epithelial cells.

4.7. Other signaling

Mitogen-activated protein kinase (MAPK) signaling plays a key role in cell growth, proliferation, migration and apoptosis. Modulation of MAPK signaling by ethanol is distinctive, depending on the context (cell type, acute or chronic exposure, normal or transformed cell phenotype) [162]. Chronic ethanol treatment inhibits p42/44 MAPK and growth factor-stimulated p42/44 MAPK activation. In contrast, chronic ethanol potentiates endotoxin-stimulated p42/44 MAPK and p38 MAPK signaling in Kupffer cells. In mouse tongue epithelial cells, ethanol alone increased the expression of p38 MAPK, but not Erk1/2 MAPK [150].

PI3K/Akt signaling is a critical regulatory axis for cell growth, motility and survival [163]. The effects of ethanol on PI3K/Akt signaling vary depending on the cell type and ethanol concentration. PI3K/Akt activation was involved in acute ethanol-induced fatty liver in mice [164]. Similarly, activation of Akt was observed in the nucleus accumbens of rodents after acute systemic administration of alcohol [165]. On the other hand, PI3K/Akt signaling was inhibited by ethanol in primary cortical neurons [166]. Interestingly, low-concentration alcohol reduced, but high-concentration enhanced, Akt activity in rat cardiac tissue and cardiocytes [167].

G-protein coupled receptors (GPCRs) are involved in many biological functions, including inflammation, carcinogenesis and chemotaxis, and may cross-talk with other signaling pathways in mediating these cellular processes [168]. GPR 55 was overexpressed in monocyte-derived dendritic cells from alcohol users or those treated with ethanol [169]. The ability of ethanol to increase gamma-aminobutyric acid release can be regulated by different GPCRs, such as cannabinoid-1 receptor, corticotropin-releasing factor 1 receptor and 5-hydroxytryptamine 2C receptor [170].

5. Overall effects of ethanol on the oro-esophageal epithelium

Ethanol has been shown to cause several changes in cellular behaviors of oro-esophageal squamous epithelial cells. Treatment of human OESCC cells with ethanol enhances cell proliferation and alters expression of cytokeratins suggesting inhibition of squamous cell differentiation [32]. *In vivo*, chronic drinking of acetaldehyde causes hyperproliferation and suppresses differentiation of oro-esophageal epithelial cells [171]. Meanwhile, chronic exposure to ethanol causes epithelial atrophy and reduces basal cell size of rat esophageal mucosa [172]. These changes in cell proliferation and differentiation may be explained by a recent study showing that ethanol promotes symmetric division of stem cells in oral epithelium [150].

Chronic inflammation induced by alcohol drinking promotes carcinogenesis. In the intestine, ethanol-induced mast cell-mediated inflammation promotes tumorigenesis in a mouse model [173]. Ethanol supports macrophage recruitment and reinforces invasion and migration of Lewis lung carcinoma in mice [174]. Similarly, we observed that ethanol promoted oral carcinogenesis through activation of the 5-lipoxygenase pathway, a pro-inflammatory pathway of arachidonic acid metabolism. Angiogenesis was also promoted in 4NQO-induced mouse tongue SCC by ethanol [35].

6. Summary

Ethanol consumption clearly contributes to OESCC as both a co-carcinogen and a tumor promoter potentially via multiple mechanisms (Figure 1). Further studies are warranted to understand how these signaling pathways contribute to ethanol-associated OESCC. One challenge comes from known interactions between pathways. For example, Wnt signaling is associated with Notch signaling. Knockdown of Notch1 enhances the activation of Wnt target genes, whereas chemical inhibition of γ -secretase or expression of a Notch1 mutant inhibits Wnt signaling [175,176]. Notch signaling also interacts with Nrf2 signaling. In mouse embryonic fibroblasts, Nrf2 regulates Notch1 expression through a functional

antioxidant response element in the promoter. Nrf2 disruption impedes liver regeneration which can be rescued by reestablishment of Notch1 signaling [177].

Another challenge is lack of proper *in vivo* models. Studies on animal models have failed to demonstrate a strong promoting effect of ethanol on OESCC. In one study, life-long exposure to 10% ethanol in drink significantly increased the incidence of oral cancer in Sprague-Dawley rats [178]. Several previous studies have found that co-administration of ethanol enhanced chemically induced OESCC [73,179–181]. In the oral cavity, studies have suggested, but failed to confirm from a statistical point of view, the cancer-promoting activity of ethanol feeding or painting on 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster cheek pouch carcinogenesis [179,182,183]. In the esophagus, ethanol was reported to have only weak or no promoting activity on carcinogenesis [184–188]. Most of these studies could not reach solid conclusions with enough statistical power due to small sample sizes. Although concentrated ethanol is believed to be more effective in promoting cancer than diluted ethanol, one major limitation in animal studies is poor tolerance of lab animals to concentrated ethanol. ~20% (w/v, or 25% v/v) or ~32% (w/v, or 40% v/v) is the upper limit of ethanol concentration in drinking water for mice or rats, respectively [189]. In addition, ethanol passes the oral cavity and esophagus quickly, thus its effects on the oroesophageal squamous epithelial cells *in vivo* tend to be weak and transient. Several approaches have been used to enhance voluntary alcohol drinking in animals. Sweeteners are commonly used in ethanol self-administration paradigms, either to initiate ethanol drinking or to increase ethanol consumption [190]. A variety of studies performed in at least seven strains of rats and most inbred strains of mice indicated a close association between consumption of sweet solution (i.e. 0.2% saccharin, 5% sucrose) and ethanol intake [191–195]. Supersaccharin (0.125% saccharin and 3% sucrose) is a commonly used sweetener for rats [196]. Gradual increase of ethanol concentration in drink and using a prandial model (postmeal drinking) can also help rodents acclimate to relatively concentrated ethanol [197]. We use forestomach as the surrogate target organ site in a carcinogen-induced OESCC model based on the histological similarities between the esophagus and the forestomach [49,198].

Recently, advanced molecular techniques have been widely used in cancer research. Genomics techniques such as NextGen sequencing, CGH array and SNP array are used for detection of DNA alterations, RNA-Seq and microarray for detection of mRNA differential expression, and methylation array for detection of alterations in gene methylation [199,200]. NextGen sequencing data of ESCC show that non-alcohol users show a relatively distinct mutation pattern and have better prognosis than alcohol users [201]. Metabolomics has been used to identify biomarkers of ethanol-induced liver disease [202,203]. Microbiomics may help us understand how ethanol modifies oral bacteria and whether certain bacteria may be more carcinogenic than others [204,205]. We expect that many new discoveries will be made in our understanding of the molecular mechanisms of ethanol-associated OESCC.

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Abbreviations

4NQO	4-nitroquinoline 1-oxide
ADH	alcohol dehydrogenase
ALDH	acetaldehyde dehydrogenase
CYP2E1	cytochrome P450 2E1
Edh	EH-domain containing gene
ELOVL	very long chain fatty acid elongase
GPCRs	G-Protein coupled receptors
HNE	4-hydroxynonenal
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
N²-EtidG	N ² -ethylidene-2'-deoxyguanosine
NFκB	nuclear factor κB
NICD	Notch intracellular domain
Nrf2	nuclear factor erythroid 2-like factor
OESCC	oro-esophageal squamous cell carcinoma
PI3K	phosphoinositide 3-kinase
RBPJ	recombining binding protein for immunoglobulin kappa j
ROS	reactive oxygen species
RR	relative risk
SCC	squamous cell carcinoma
Shh	sonic hedgehog
TGFβ	transforming growth factor beta
TLR4	Toll-like receptor 4

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Highlights

- Alcohol drinking is a major etiological factor of oro-esophageal squamous cell carcinoma.
- Mechanisms of ethanol-associated oro-esophageal squamous cell carcinoma are reviewed.
- Disturbance of systemic metabolism of nutrients, of redox metabolism and of signaling pathways in squamous epithelial cells are proposed mechanisms.

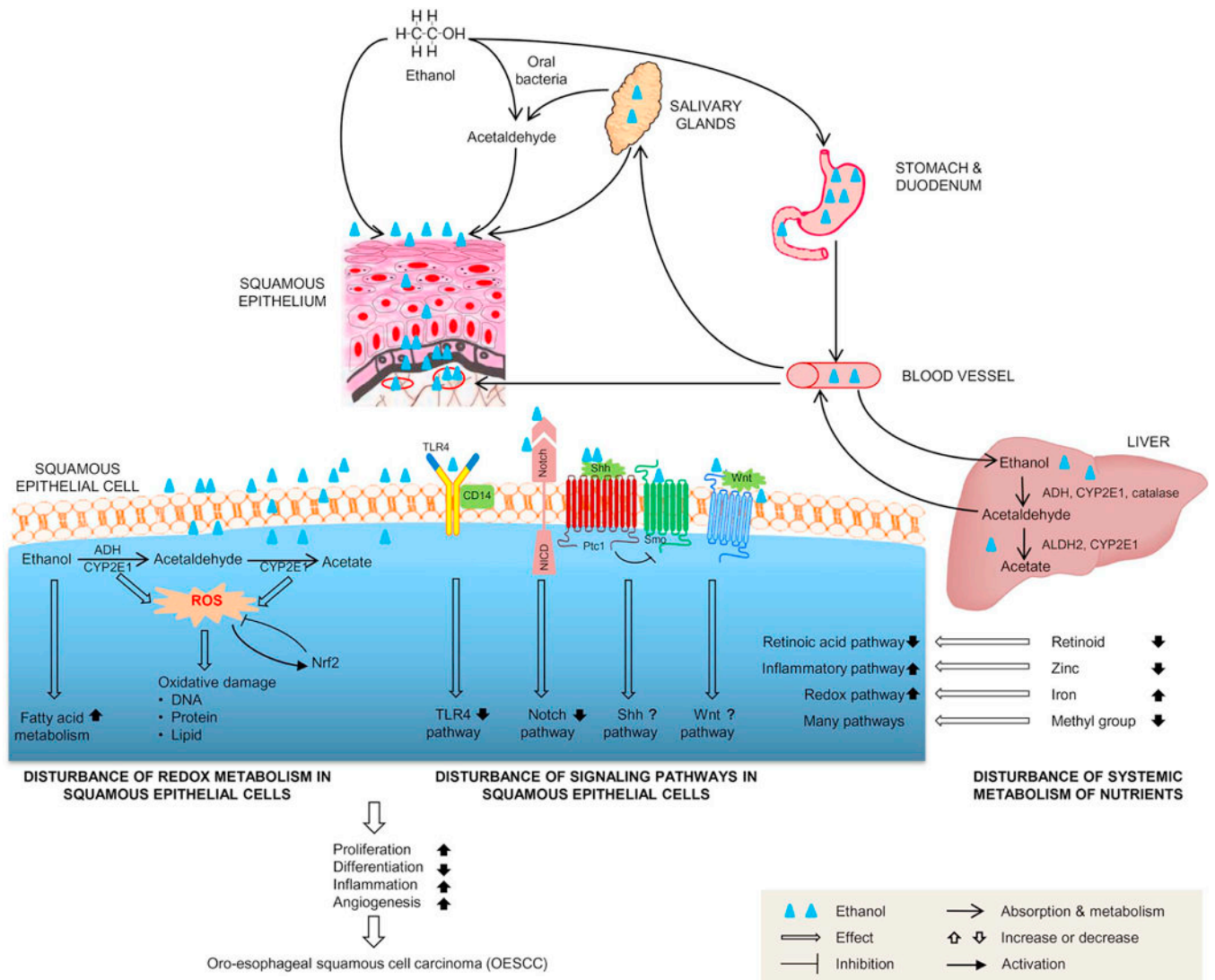


Figure 1.

Molecular mechanisms of ethanol-associated OESCC. After ingestion, ethanol is quickly absorbed through the stomach and duodenum into blood stream, and metabolized mainly in the liver. Ethanol is oxidized into acetaldehyde by ADH, CYP2E1 and catalase, and then into acetate by ALDH. Systemic metabolism of ethanol causes reduction of retinoid, zinc and methyl group and accumulation of iron. Through the blood stream, ethanol is circulated to the oro-esophageal epithelium and salivary glands. In the saliva, ethanol is oxidized by microbes to acetaldehyde. When ethanol and acetaldehyde contact oro-esophageal epithelial cells, ethanol perturbs the lipid bilayer of cell membrane and interferes the function of intrinsic membrane proteins, such as TLR4, Notch, Shh and Wnt pathway receptors, and thus activates or inhibits downstream signaling. Inside the epithelial cells, ethanol metabolism causes oxidative damages to DNA, proteins and lipids, and modulates fatty acid metabolism as well. These systemic and local effects of ethanol and acetaldehyde stimulate

cell proliferation, inflammation and angiogenesis, and suppress squamous cell differentiation, and therefore promote OESCC.

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