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Ethanol-mediated carcinogenesis in the human esophagus implicates CYP2E1 induction and the generation of carcinogenic DNA-lesions

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Chronic alcohol consumption is a major risk factor for esophageal cancer. Various mechanisms may mediate carcinogenesis including the genotoxic effect of acetaldehyde and oxidative stress. Ethanol exerts its carcinogenic effect in the liver among others *via* the induction of cytochrome P450 2E1 (CYP2E1) and the generation of carcinogenic etheno-DNA adducts. Here we investigated if such effects can also be observed in the human esophagus. We studied nontumorous esophageal biopsies of 37 patients with upper aerodigestive tract cancer and alcohol consumption of 102.3 ± 131.4 g/day (range: 15–600 g) as well as 16 controls without tumors (12 teetotalers and 4 subjects with a maximum of 25 g ethanol/day). CYP2E1, etheno-DNA adducts and Ki67 as a marker for cell proliferation were determined immunohistologically. Chronic alcohol ingestion resulted in a significant induction of CYP2E1 ($p = 0.015$) which correlated with the amount of alcohol consumed ($r = 0.6$, $p < 0.001$). Furthermore, a significant correlation between CYP2E1 and the generation of the carcinogenic exocyclic etheno-DNA adducts 1,N^6-ethenodeoxyadenosine ($r = 0.93$, $p < 0.001$) and 3,N^4-ethenodeoxycytidine ($r = 0.92$, $p < 0.001$) was observed. Etheno-DNA adducts also correlated significantly with cell proliferation ($p < 0.01$), which was especially enhanced in patients who both drank and smoked ($p < 0.001$). Nonsmokers and nondrinkers had the lowest rate of cell proliferation, CYP2E1 expression and DNA lesions. Our data demonstrate for the first time an induction of CYP2E1 in the esophageal mucosa by ethanol in a dose dependent manner in man and may explain, at least in part, the generation of carcinogenic DNA lesions in this target organ.

Carcinogenesis

Chronic alcohol consumption is a major health issue worldwide and may account for ~1.8 million deaths per year (3.2% of all deaths).[1] One of the most significant diseases caused by chronic alcohol consumption is cancer. Recently, the International Agency for Research on Cancer (IARC) has evaluated the role of ethanol in carcinogenesis and has concluded that the occurrence of malignant tumors of the upper aerodigestive tract (oral cavity, pharynx, larynx, esophagus) the liver, the colorectum and the female breast are causally related to the consumption of alcoholic beverages.[2,3] Worldwide, a total of approximately 389,000 cases of cancer representing 3.6% of all cancers derive from chronic alcohol consumption.[1]

Worldwide epidemiologic studies have identified alcohol as the most important risk factor for esophageal cancer.[2,4–10] This effect is dose dependent[4] and smoking in addition to alcohol consumption leads to synergistic effects in carcinogenesis.[11,12] A carefully designed study in French patients demonstrated that an alcohol consumption of more than 80 g/day is associated with a relative risk (RR) of 18 for the development of esophageal carcinoma, while smoking 20 cigarettes a day resulted in an increased risk of about 5. However, both factors act synergistically, resulting in an increased RR of 44.[13]

Various mechanisms may explain the carcinogenic effect of alcohol on the esophagus including the genotoxic action of acetaldehyde, the first and most toxic metabolite of

Key words: esophageal cancer, alcohol, DNA adducts, oxidative stress, cytochrome P4502E1

Abbreviations: BSA: bovine serum albumin; CYP2E1: cytochrome P450 2E1; DAB: diaminobenzidine; 4-HNE: 4-hydroxy-2-nonenal; IARC: International Agency for Research on Cancer; LPO: lipid peroxidation; MDA: malondialdehyde; PBS: phosphate-buffered saline; ROS: reactive oxygen species; RR: relative risk; εdA: 1,N^6-ethenodeoxyadenosine; εdC: 3,N^4-ethenodeoxycytidine

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ethanol and ethanol-mediated oxidative stress reactions.[6,14] It is widely recognized that reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) play an important role in alcohol-induced liver injury and in hepatocarcinogenesis.[6,15] Chronic ethanol consumption results in the generation of ROS *via* multiple pathways leading to lipid peroxidation (LPO) and LPO-products such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). These DNA-reactive aldehydes in turn form mutagenic exocyclic DNA adducts including 1,N^6-ethenodeoxyadenosine (εdA) and 3,N^4-ethenodeoxycytidine (εdC).[16–18] These etheno DNa adducts have been shown to promote carcinogenesis.[19]

Several enzyme systems are capable of producing ROS including the mitochondrial respiratory chain, the cytosolic enzymes xanthine oxidase and aldehyde oxidase, as well as the microsomal cytochrome P450-dependent mono-oxygenases.[15] Among the latter, cytochrome P450 2E1 (CYP2E1) is involved in alcohol-mediated generation of oxidative stress.[6] CYP2E1 is induced among other factors by its substrate ethanol. Debate still exists as to the precise mechanism of CYP2E1 induction by ethanol. There is some evidence that CYP2E1 is stabilized by its substrates at the protein level.[20,21] RNA-stabilization known to play a role in acetone-mediated induction in diabetic rats[21] does not seem to be relevant in ethanol-mediated regulation.[21] Transcriptional interference with HNF1α (hepatocyte nuclear factor 1α) that regulates CYP2E1 expression on transcriptional level has been ruled out.[22]

Induction of CYP2E1 has been shown to correlate significantly with the generation of hydroxyethyl radicals and with LPO-products such as 4-HNE and MDA.[23] CYP2E1 is induced by chronic alcohol consumption within a week even at a relatively low ethanol dose (40 g/d), but the degree of CYP2E1 induction varies substantially between individuals.[24] An increase of oxidative DNA adducts and of mutagenic apurinic and apyrimidinic DNA sites were found in wild-type mice chronically fed ethanol, but not in mice knocked out for functional CYP2E1 further supporting the role of CYP2E1 in the generation of DNA damage after ethanol ingestion.[25] Recently, we have been able to detect etheno-DNA adducts such as εdA in livers of patients with alcoholic liver disease.[26,27] In rats, *N*-nitrosomethylbenzylamine-induced esophageal carcinogenesis has been shown to be associated with increased levels of CYP2E1.[28]

The aim of our study was to determine if (*i*) chronic ethanol consumption induces CYP2E1 in the human esophageal mucosa similarly as in the liver, (*ii*) a correlation exists between CYP2E1 expression and the occurrence of exocyclic etheno-DNA adducts in the esophagus and (*iii*) such changes are associated with altered cell proliferation.

To address these questions we used nontumorous esophageal biopsies from patients with cancer of the upper aerodigestive tract. Since ethanol-mediated pathomechanisms of cancer development at these sites seem to be similar, the esophageal mucosa may therefore be a representative tissue for analysis.

Table 1. Patients' characteristics, proliferation index, cytochrome P-4502E1 intensity and etheno DNA adducts in esophageal biopsies

	Nonsmokers/ nondrinkers	Smokers, drinkers, smokers/ drinkers
	n = 11	n = 42
	3m, 8f	37m, 5f
Mean age	70 ± 11	60 ± 10
Patients with tumors	1	36
Mean alcohol consumption [g/d]	0	137 ± 59
Mean cigarette consumption [py]	0	32 ± 10
Mean proliferation rate	22 ± 16	38 ± 13
Median CYP2E1 staining intensity	0 (range 0–2)	2 (range 0–3)
Mean % of edA positive nuclei	0.8 ± 1.1	33.1 ± 23
Mean % of edC positive nuclei	0.4 ± 0.7	32.8 ± 25.4

Material and Methods

Human esophageal biopsy specimen

In our study, we included a total of 53 patients (40 male/13 female). The mean age was 61 ± 11 years. Thirty-seven of them have been diagnosed with a tumor of the upper aerodigestive tract, 16 had no history of malignancy (Table 1). Fourteen patients were teetotalers, 14 consumed between 1 and 60 g ethanol per day and 25 were heavy drinkers with more than 60 g ethanol per day. A total of 16 patients were smokers with a mean consumption of 33 ± 13 packs per year. Patient's characteristics are given in Table 1.

After obtaining informed consent, patients underwent routine upper gastrointestinal endoscopy including chromoendoscopy using 2% Lugol's iodine solution to check for precancerous lesions. Biopsies were taken from Lugol-unstained lesions if present and from stained tissue in case of homogeneous iodine staining, and fixed in formaldehyde or snap-frozen in liquid nitrogen for further testing.

The study was approved in accordance with the declarations of Helsinki by the Ethics Committee of the University Schleswig-Holstein in Lübeck.

Immunohistochemical staining for Ki67

Paraffin-embedded tissue was dewaxed in xylene, rehydrated in a decreasing ethanol series and finally washed in distilled water. Antigen retrieval was performed by microwaving the slides in citrate buffer (10 mM, pH6) for 6 min at 800 W, for 9 min at 150 W and a third time for 15 min at 150 W. Slides were slowly cooled down to room temperature. The primary antibody was applied over night at 4°C (p53 [Progen, Heidelberg, Germany] 1:250 in TBS (tris-buffered saline, pH 7.4), Ki67 [DAKOCytomatation, Glostrup, Denmark]

1:75 in TBS). After washing the slides in TBS for 10 min, the secondary antibody (rabbit-anti-mouse Ig [DAKOCytomatation, Glostrup, Denmark] 1:25) was incubated for 30 min at room temperature, followed by another washing step and incubation with the APAAP-complex [DAKOCytomatation, Glostrup, Denmark] 1:50 in TBS for another 30 min at room temperature. Incubation with secondary antibody and APAAP-complex were repeated twice in order to obtain intense staining. Finally, staining was developed in a chromogenic solution containing Fast Red (Sigma, Taufkirchen, Germany). Sections were then counterstained in Mayer's Hemalaun and mounted in glycerin gelatine.

Immunohistochemical staining of CYP2E1

Frozen esophageal biopsy samples were cut into 6 μm sections, placed on 3-aminopropyl-triethoxysilan-coated glass microscope slides, fixed in acetone at −20°C for 10 min and air-dried.

Sections were treated with 0.5% H_2O_2 in absolute methanol for 10 min to quench endogenous peroxidase activity. Thereafter, sections were incubated for 2 hr at room temperature with the primary antibody (rabbit antihuman CYP2E1, 1:100, Chemicon, Hofheim, Germany) and 5% normal goat serum to block nonspecific binding. VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA) was used for detection according to the manufacturer's protocol. Staining was developed by incubating the sections for 5 min in diaminobenzidine (DAB). Sections were counterstained with hematoxylin and mounted in Aquatex mounting medium. Negative controls were performed by omitting the primary antibody.

Immunohistochemical detection of etheno-DNA adducts

Staining was performed with 4 μm cryotome sections of esophageal biopsies using the method developed in our laboratory.[26,29] Acetone-fixed slides were dipped in phosphate-buffered saline (PBS) for 10 min, and then placed in 0.3% H_2O_2 in absolute methanol for 10 min to quench endogenous peroxidase. Slides were incubated with proteinase K (20 μg/ml) (Roche, Mannheim, Germany) in double distilled H_2O at room temperature for 10 min to remove histone and nonhistone proteins from DNA increasing antibody accessibility. After washing with PBS, slides were treated with 100 μg/ml RNAse (Roche, Mannheim, Germany) (heated for 10 min at 80°C to inactivate DNAse) at 37°C for 1 hr to prevent antibody binding to RNA adducts and then washed in PBS. To denature DNA, cells were treated with 1.5N HCl for 5 min at room temperature and subsequently rinsed in PBS and double distilled water. The pH was neutralized with 50 mM Trisbase buffer, pH 7.4 for 5 min at room temperature. Nonspecific binding sites were blocked with 8% bovine serum albumin (BSA), 2% horse serum, 0.05% Tween and 0.05% Triton X-100 for 20 min at room temperature. Slides were incubated with the primary monoclonal antibody EM-A-1 against εdA and EM-C-1 against εdC (provided by Drs. P. Lorenz and M. Rajewsky, University of Essen, Essen, Germany), at a dilution of 1:20) at 4°C overnight.[29,30] After washing with PBS, the antibody detection was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. DAB was used as a chromogen to visualize the reaction. The reaction was stopped after 5 min with H_2O. Slides were counterstained with DAPI and mounted with Kaiser's glycerine–gelatine. All slides were subjected to the same standardized conditions. Negative controls were performed by omitting the primary antibody.

Imaging and semiquantitative analysis of immunohistochemical staining

Representative pictures were taken at a magnification of 200× with a Leica Image Manager 50 (Leica, Solms, Germany) and analyzed using Image J software (Toronto Western Research Institute, UK). The proliferation rate is given as % Ki67-positive nuclei; the frequency of εdA and εdC positively stained nuclei was expressed as % of stained cell nuclei over total number of cells counted.

The staining intensity for CYP2E1 was assessed according to the scale devised by Tsutsumi *et al.*,[31] whereby 3+, 2+, 1+ and 0 denote intense, moderate, slight and no specific immunostaining, respectively.

Statistical analysis

Significant differences were calculated using the Mann-Whitney-U-test for nonparametric parameters. Correlations were calculated by Spearman rank analysis. A p-value < 0.05 was considered significant. All statistical analyses were performed with SPSS, version 12.0.1 (SPSS, Munich, Germany).

Results

In our study, we included a total of patients (40 male/13 female). The mean age was 61 ± 11 years. Thirty-seven of them have been diagnosed with a tumor of the upper aerodigestive tract, 16 had no history of malignancy (Table 1). Fourteen patients were teetotalers, 14 consumed between 1 and 60 g ethanol per day and 25 were heavy drinkers with more than 60 g ethanol per day. Sixteen patients were smokers with a mean consumption of 33 ± 13 packs per year. Patient's characteristics and individual results in immunohistochemistry are given in Table 1.

Smokers who drink have a significantly higher proliferation rate, higher CYP2E1 expression and more DNA-lesions

There was a highly significant difference between patients who smoke and drink alcohol compared to those who did not. Patients smoke and drink (more than 10 pack years and more than 10 g ethanol/day) had a significantly higher proliferation rate ($p = 0.015$) (Fig. 1*a*), CYP2E1 expression ($p < 0.001$) (data not shown) and a higher prevalence of nuclei

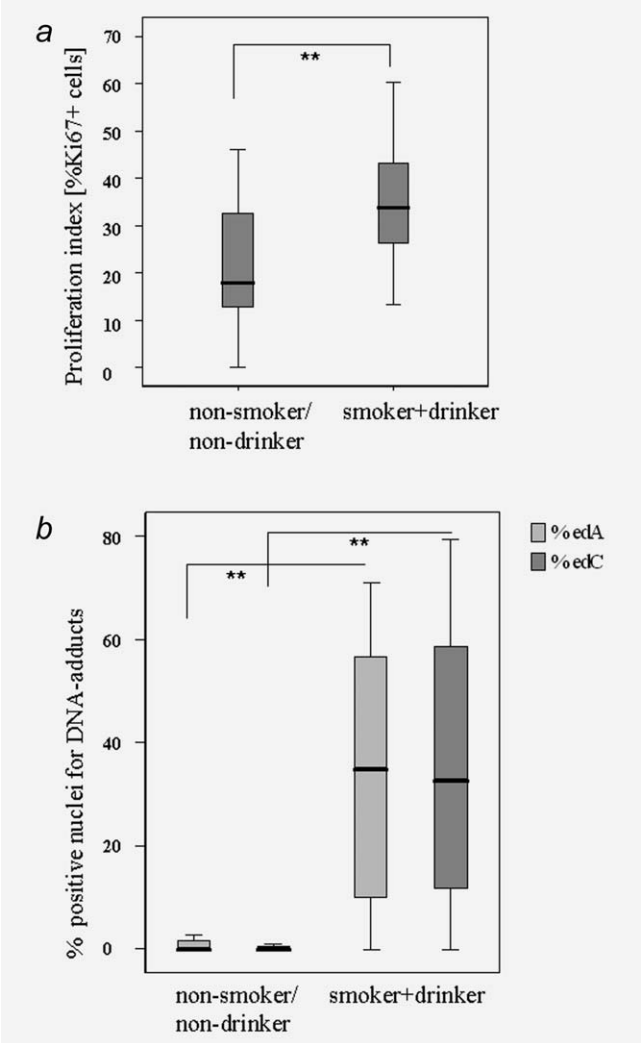

Figure 1. Effect of drinking and smoking on esophageal cell proliferation (*a*) and exocyclic DNA adduct formation (*b*) presented as Box plots. Smoking in combination with alcohol drinking significantly increases proliferation rate (*a*) and DNA-lesions (*b*) in the esophageal mucosa (**$p < 0.001$). Proliferation rate was determined by staining for Ki67 and calculating the percent of positive nuclei in the esophageal mucosa. Etheno-DNA lesions were determined by immunohistochemistry for εdA and εdA followed by counting of the percentage of positively stained nuclei.

containing etheno-DNA-lesions ($p < 0.001$ for εdA and εdC) than those who did not (Fig. 1*b*).

Patients with cancer show significantly increased proliferation rate, CYP2E1-expression and etheno-DNA adducts in esophageal mucosa

Patients with tumors showed a significantly higher proliferation rate ($p < 0.001$) (Fig. 2), CYP2E1 staining intensity ($p = 0.04$) and prevalence of nuclei positive for εdA ($p = 0.01$) and εdC ($p = 0.01$) compared to patients without upper aerodigestive tract cancer. Representative esophageal biopsies stained for CYP2E1 and DNA-lesions are shown in Figure 3.

Alcohol consumption correlates with CYP2E1-expression and the presence of etheno-DNA-adducts but not proliferation rate

Calculating bivariate correlations (Spearman rank) between alcohol consumption and investigated markers showed that there are a strong positive correlations between alcohol consumption, CYP2E1 staining intensity ($r = 0.6$, $p < 0.001$) and the prevalence of nuclei positive for εdA ($r = 0.68$, $p < 0.001$) and εdC ($r = 0.68$, $p < 0.001$).

Patients who showed induction of CYP2E1 were most likely to develop DNA-lesions (Fig. 3). There was a strong correlation between staining intensity for CYP2E1 and εdA ($r = 0.93$, $p < 0.001$) and εdC ($r = 0.92$, $p < 0.001$).

In contrast to smoking expressed as pack years, alcohol consumption did not correlate with proliferation rate ($p < 0.001$ for pack years *vs.* $p = 0.16$ for alcohol).

Age has no influence on proliferation rate or DNA-damage

In our patient population, there was no apparent association of age with any tested marker.

Discussion

Patients with cancers of the upper aerodigestive tract are predominantly smokers and drinkers. From Asian patients, we know that increased circulating levels of acetaldehyde due to a less active acetaldehyde dehydrogenase (AcDH*2) increase the risk of esophageal carcinoma.[2,6,32,33] Since CYP2E1 is also involved in acetaldehyde generation leading to increased levels of LPO byproducts and DNA-damage, we investigated whether markers for this process can be found in esophageal mucosa of patients with cancers of the upper aerodigestive tract.

CYP2E1 is present in the esophageal mucosa. Others have shown that patients with esophageal squamous cell carcinoma showed increased expression of CYP2E1 in the esophageal mucosa compared to patients without such tumors. However, there was no difference in CYP2E1 expression within the same patient between malignant and surrounding tissue.[34]

In our study, increased CYP2E1 expression in the esophageal mucosa of patients with upper aerodigestive tract cancer closely correlated with prevalence of nuclei positive for εdA and εdC. The mechanism that leads to these etheno-DNA lesions is thought to involve CYP2E1-mediated ROS-generation leading to LPO and DNA adduct formation.[16–18,27] The major LPO-product 4-HNE and MDA can react with DNA bases generating exocyclic -DNA adducts. While MDA forms an exocyclic propano adduct with the *N*-1 and N^2-position of guanine (M1dG), 4-HNE induces DNA damage through its putative epoxidation-product, 2,3-epoxy-4-hydroxynonanal).[16,35] Substituted 1,N^2-ethenoguanine, 1,N^2-ethenoguanine, 3,N^4-ethenocytosine and 1,N^6-ethenoadenine have been

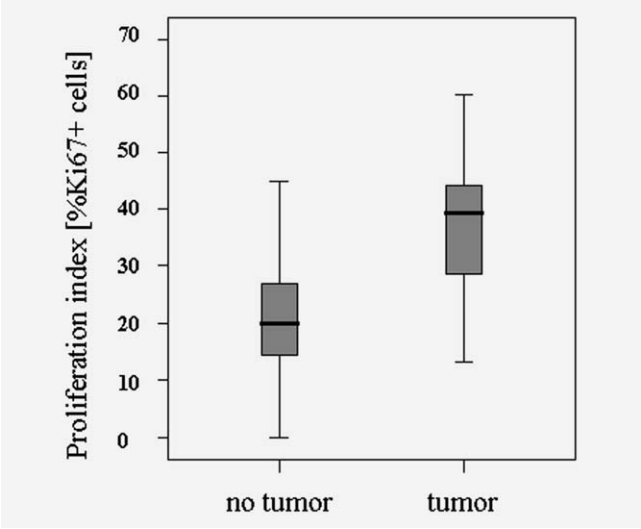

Figure 2. Influence of tumor presence on esophageal cell proliferation presented as Box plot. Patients with upper aerodigestive malignancies showed an increased proliferation rate compared to control patients without cancer (**$p < 0.001$). Proliferation rate was determined by staining for Ki67 and calculating the percent of positively stained nuclei in the esophageal mucosa.

characterized as reaction products *in vitro*, the most prevalent adduct being 1,N^2-etheno-2′-deoxyguanosine.[36–38] In our study, we have now identified these carcinogenic etheno-DNA lesions in the esophagus, a major target organ for alcohol-mediated carcinogenesis.

It has to be emphasized that the exocyclic etheno DNA-adducts studied here are distinct from the types of DNA-adducts generated from exogenous carcinogens, *e.g.*, present in tobacco smoke. LPO-derived exocyclic-DNA adducts are believed to be formed from inflammatory-driven oxidative stress reactions.[39,40] Increased accumulation of LPO-by-products such as 4-HNE and oxidized proteins are critical features in ethanol-induced liver injury.

Excess levels of etheno-adducts have been detected in chronically inflamed or infected organs of cancer prone patients.[18] Exocyclic nucleobase adducts exhibit pro-mutagenic properties producing base pair substitution mutations and apparently are poorly repaired in some tissues and cells.[16,41–43] Of considerable biological importance is also the finding that inflammatory mediators, including 4-HNE impair the repair of exocyclic etheno DNA lesions and of bulky exogenous carcinogen(*e.g.*, benzo(a)pyrene) adducts in human cells.[44]

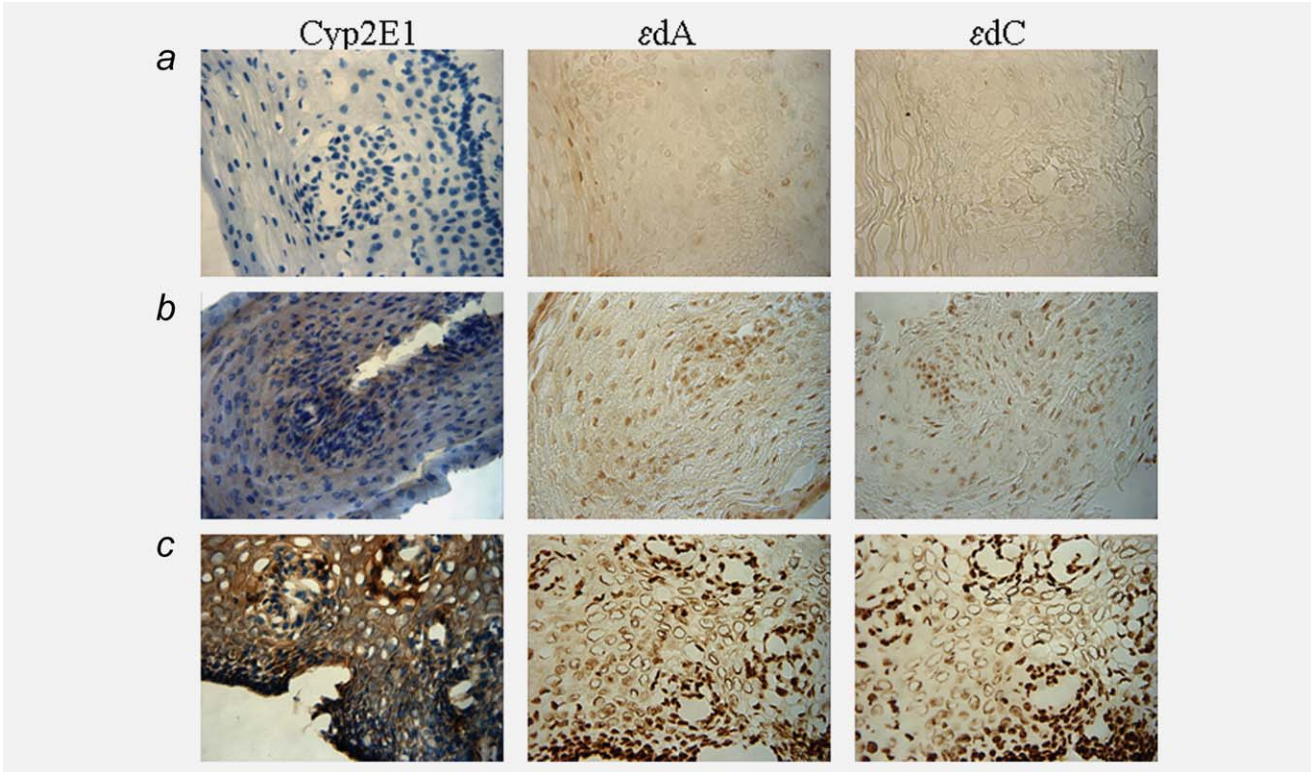

Figure 3. Alcohol consumption correlates significantly with expression of CYP2E1 and the number of nuclei positive for DNA-lesions (εdA and εdC, respectively). Row A shows esophageal biopsies of a healthy control with almost no CYP2E1 expression and no DNA-lesions. Row B represents nontumorous esophageal biopsies of a patient with oral carcinoma, a daily ethanol consumption of 112 g and 30 pack-years. Row C represents a biopsy from a patient with larynx carcinoma, a daily ethanol consumption of 260 g and 50 pack-years. (Horseradish peroxidase staining, counterstaining with Hematoxylin, magnification 400×). Biopsies B and C were taken from Lugol-unstained lesions.

For all these reasons, etheno-DNA adducts probably play a causal role in the initiation and progression of carcinogenesis particularly when chronic inflammatory processes are part of the etiopathogenesis.[18]

Although alcohol consumption correlates significantly with CYP2E1 expression and DNA-lesions in the esophageal biopsies, this correlation is not nearly as strong as the correlation between CYP2E1 expression and DNA-lesions. This can be explained by the observation that the individual alcohol threshold to induce CYP2E1 expression varies interindividually.[24]

It should be emphasized that acetaldehyde has been recognized as a carcinogenic factor in esophageal carcinogenesis since individuals who accumulate acetaldehyde after alcohol consumption have a significant increased cancer risk.[31,32] Acetaldehyde (generated by alcohol dehydrogenase and by CYP2E1) may not only bind to DNA forming carcinogenic acetaldehyde-DNA adducts, it does also damage the antioxidative defense system including glutathione which is responsible for the destruction of ROS generated *via* CYP2E1.[6] Thus, acetaldehyde and ROS may act in concert enhancing the carcinogenic action of each other.

Surprisingly in our study, we could not find a significant effect of ethanol on esophageal cell proliferation. This is in contrast to the data of Simanowski *et al.*[45] from our laboratory, who found an increased esophageal cell proliferation in rats after chronic ethanol administration as Lieber-DeCarli diet. This increase in cell proliferation after alcohol administration however disappeared when the salivary glands were surgically removed underlining the fact that obviously saliva is necessary to initiate such an effect. It has been shown that chronic ethanol consumption results in morphological and functional damage of the salivary glands modifying saliva flow.[46] Such an effect in our patients could explain the lack of correlation between alcohol intake and mucosal regenerativity in our study.

On the other hand in our study, smoking as well as both smoking and drinking resulted in a significant enhancement of cell proliferation. Tobacco smoke may enhance cell proliferation and carcinogenesis by different mechanisms. CYP2E1 induction by ethanol may result in an enhanced activation of various pro-carcinogens present in tobacco smoke.[47] This interaction between alcohol and tobacco carcinogens has been studied by Farinati *et al.*[48] who showed in a rat model an enhanced activation of the tobacco carcinogen *N*-nitrosopyrrolidine after CYP2E1-induction by chronic alcohol administration.

As the esophageal mucosa contains additionally a wide variety of cytochrome P-450 species, including CYP1A, CYP3A, CYP4A as well as CYP4B1, CYP2B6/7 and CYP2C this tissue has the capacity to activate a wide range of chemical carcinogens including several of those present in tobacco smoke into reactive DNA binding metabolites.[49–51]

It is interesting to note that in our study DNA lesions were shown in the esophageal mucosa, *i.e.*, outside of the place where the cancer developed. This shows that the whole area of the upper aerodigestive tract is at a higher risk of cancer development. Screening patients with head and neck cancer for other premalignant or malignant lesions might thus be important and one could speculate that etheno-DNA-adducts might be used in high risk patients (smokers and drinkers) as a marker to intensify endoscopic surveillance.

In conclusion, we demonstrated here for the first time a significant correlation between the amount of alcohol consumed, CYP2E1 protein levels and the formation of carcinogenic exocyclic etheno-DNA adducts in the esophageal mucosa of humans. Thus, the oxidative stress generated by CYP2E1-induction and resulting DNA damage in the esophageal mucosa after chronic ethanol consumption may contribute, at least in part to the mechanisms of ethanol-mediated esophageal carcinogenesis.

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