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 immunohistochemically. 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,N6-ethenodeoxyadenosine (r = 0.93, p < 0.001) and 3,N4-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.

Key words: esophageal cancer, alcohol, DNA adducts, oxidative stress, cytochrome P450 2E1

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; zDA: 1,N6-ethenodeoxyadenosine; zDC: 3,N4-ethenodeoxycytidine

G.M. and Y.W. contributed equally to this work

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ethanol and ethanol-mediated oxidative stress reactions.\textsuperscript{6,14} It is widely recognized that reactive oxygen species (ROS) such as superoxide anion (O\textsuperscript{2−}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) play an important role in alcohol-induced liver injury and in hepatocarcinogenesis.\textsuperscript{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\textsubscript{6}-ethenodeoxyadenosine (\textit{edA}) and 3,N\textsubscript{4}-ethenodeoxycytidine (\textit{edC}).\textsuperscript{16–18} These etheno DNA adducts have been shown to promote carcinogenesis.\textsuperscript{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.\textsuperscript{15} Among the latter, cytochrome P450 2E1 (CYP2E1) is involved in alcohol-mediated generation of oxidative stress.\textsuperscript{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.\textsuperscript{20,21} RNA-stabilization known to play a role in acetone-mediated induction in diabetic rats\textsuperscript{21} does not seem to be relevant in ethanol-mediated regulation.\textsuperscript{21} Transcriptional interference with HNF1\textsubscript{a} (hepatocyte nuclear factor 1\textsubscript{a}) that regulates CYP2E1 expression on transcriptional level has been ruled out.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{25} Recently, we have been able to detect etheno-DNA adducts such as \textit{edA} in livers of patients with alcoholic liver disease.\textsuperscript{26,27} In rats, N-nitrosomethylbenzylamine-induced esophageal carcinogenesis has been shown to be associated with increased levels of CYP2E1.\textsuperscript{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.

**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 overnight at 4°C (p53 [Progen, Heidelberg, Germany] 1:250 in TBS (tris-buffered saline, pH 7.4), Ki67 [DAKOCytmotation, Glostrup, Denmark]

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<th>Table 1. Patients’ characteristics, proliferation index, cytochrome P-4502E1 intensity and etheno DNA adducts in esophageal biopsies</th>
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Acetone-fixed slides were dipped in phosphate-buffered saline for 10 min at room temperature, followed by another washing step and incubation with the APAAP-complex (DAKO, Glostrup, Denmark) for 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 Hemalun 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% H2O2 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 diamobenzidine (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. Acetone-fixed slides were dipped in phosphate-buffered saline (PBS) for 10 min, and then placed in 0.3% H2O2 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 H2O 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 ɛdA and EM-C-1 against ɛdC (provided by Drs. P. Lorenz and M. Rajewsky, University of Essen, Essen, Germany), at a dilution of 1:20 at 4°C overnight. 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 H2O. 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 ɛdA and ɛdC 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., 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. 1a), CYP2E1 expression (p < 0.001) (data not shown) and a higher prevalence of nuclei
containing etheno-DNA-lesions \( (p < 0.001) \) for \( \text{edA} \) and \( \text{edC} \) than those who did not (Fig. 1).

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 \( (r = 0.68, p < 0.001) \) and the prevalence of nuclei positive for \( \text{edA} \) \( (r = 0.68, p < 0.001) \) and \( \text{edC} \) \( (r = 0.68, p < 0.001) \).

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 \( \text{edA} \) \( (r = 0.93, p < 0.001) \) and \( \text{edC} \) \( (r = 0.92, p < 0.001) \).

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

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.\(^2\)\(^{\text{a}}\)\(^{\text{,}}\)\(^{\text{32,33}}\) 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.\(^3^4\)

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 \( \text{edA} \) and \( \text{edC} \). The mechanism that leads to these etheno-DNA lesions is thought to involve CYP2E1-mediated ROS-generation leading to LPO and DNA adduct formation.\(^1^6\)\(^{-}\)\(^{18,27}\) 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-hydroxynonalal.\(^1^6\)\(^,\)\(^{35}\) Substituted 1,\(^N^2\)-ethenoguanine, 1,\(^N^2\)-ethenoguanine, 3,\(^N^4\)-ethenocytosine and 1,\(^N^6\)-ethenoadenine have been
characterized as reaction products in vitro, the most prevalent adduct being 1,N2-etheno-2'-deoxyguanosine. 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. 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. Exocyclic nucleobase adducts exhibit pro-mutagenic properties producing base pair substitution mutations and apparently are poorly repaired in some tissues and cells. 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.

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.

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 inter-individually.74

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 also damages 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.37 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.19–51

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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