GENETICS AND CELL BIOLOGY

Manganese Superoxide Dismutase (MnSOD) Polymorphism, Alcohol, Cigarette Smoking and Risk of Oesophageal Cancer

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Abstract — Aims: Alcohol, tobacco smoke and Barrett’s oesophagus as a consequence of gastro-oesophageal reflux are the main risk factors in oesophageal carcinogenesis. All risk factors may induce oxidative stress. Manganese superoxide dismutase (MnSOD) is an important repair enzyme for reactive oxidative stress (ROS)-induced damage. MnSOD polymorphisms in the −9 position of the signal sequence of the protein may lead to critical enzyme deficiency. The aim of the present study was to investigate the role of polymorphisms of MnSOD in patients with oesophageal cancer \( n = 170 \), 61 patients with adenocarcinoma (AC), 109 patients with squamous cell carcinoma (SCC) compared to heavy drinkers \( n = 160 \) and healthy blood donors \( n = 400 \). Methods: Genotyping was performed by PCR-RFLP analysis using genomic DNA extracted from whole blood. Results: The Ala/Ala genotype was 27.7% in cancer patients \( (29.5\% \text{ AC, 26.6}\% \text{ SCC}) \), 23.1% in patients with heavy alcohol use and 12.5% in the group of healthy blood donors. These results were not statistically significant after multivariate analysis controlling for age, sex, alcohol, cigarettes and interactions (odds ratio 0.92, 95% confidence interval \( = 0.63–1.36 \), for cancer patients versus heavy drinkers; odds ratio 1.02, 95% confidence interval \( = 0.51–2.03 \), for cancer patients versus blood donors; analysis by logistic regression). Subjects with an Ala/Ala genotype \( (81.3 \text{ g/day}) \) had a significantly higher alcohol intake than those with Val/Ala \( (63.9 \text{ g/day}) \) or Val/Val \( (53.8 \text{ g/day}) \) genotype \( (P = 0.00001 \) by the Kruskal–Wallis test). Conclusions: MnSOD polymorphisms play no role in the genetic predisposition to oesophageal cancer. However, our data suggest a complex gene-to-phenotype interaction between the MnSOD genotype and alcohol misuse.

INTRODUCTION

The preponderance of data from epidemiological studies links the ingestion of alcoholic beverages to an increased susceptibility to certain kinds of malignancy, such as pharyngeal, laryngeal and oesophageal cancer (Pöschl and Seitz, 2004). Oesophageal cancer is the sixth most common cancer worldwide. Chemical carcinogens, alcohol and tobacco in particular, play a major role in the pathogenesis of oesophageal squamous cell carcinoma (SCC). The major risk factor for adenocarcinoma (AC) is the development of intestinal metaplasia (Barrett’s syndrome) as a consequence of long-lasting gastro-oesophageal reflux, with a still unclear association with tobacco and alcohol misuse. Simultaneously, the observation of familial clustering of SCC as well as AC patients in high incidence regions provides another clue to a genetic background in the development of the disease (Zhang et al., 2000). One possible common molecular pathomechanism in oesophageal carcinogenesis is oxidative stress. Oxidative stress is an imbalance between prooxidant production and antioxidant defence systems. Numerous studies have attempted to establish a relationship between levels of oxidative DNA damage and cancer (Halliwell and Aruoma, 1991; Valko et al., 2004). An environment characterized by a low concentration of antioxidant enzymes and a high rate of ROS generation is deemed to lead to elevated levels of damage (Toyokuni et al., 1995). Reactive oxidative species (ROS) are produced by normal cellular respiration and as a result of inflammation and cellular stress (Oberley and Oberley, 1997). Free radicals have been implicated in a number of major disease processes, including chronic inflammation, carcinogenesis and atherosclerosis. Oxidative mechanisms have been demonstrated to possess a potential role in the initiation, promotion and progression of carcinogenesis.

Among the various defence mechanisms developed by aerobic organisms to counteract oxidative stress, antioxidant enzymes, such as superoxide dismutase (SOD), constitute the secondary defence system against endogenous as well as exogenous oxidative stress. There are three isoforms of SOD in human cells: copper–zinc SOD, found predominantly in the cytoplasm, SOD that is localized to the plasma membrane or can be secreted in the extracellular matrix removing superoxide anion from either side of the plasma membrane and MnSOD that is primarily localized in the mitochondrial matrix. MnSOD is synthesized in the cytosol and posttranscriptionally modified for transport into the mitochondrion (Wispe et al., 1989). It is well known that most of the intracellular oxygen is metabolized at the mitochondrial level. Subsequently, the mitochondrial electron transport chain is a principal source of endogenous ROS generation. As the sole intramitochondrial superoxide anion scavenger, the level of MnSOD will influence the cell’s ability to counteract ROS-induced oxidative damage. Accordingly, MnSOD polymorphisms, leading to absolute or relative deficiencies of critical enzymes within specific cellular compartments, would be one possible genetic mechanism influencing the ROS-concentration deriving from various sources. The mitochondrial targeting sequence (MTS) readily forms an amphiphilic helical structure, which is crucial for the effective transport and processing of mitochondrial proteins. A structural mutation, a T to C substitution, was found leading to an amino acid substitution at −9 position in the signal peptide from valine (Val) to alanine (Ala) (Rosenblum et al., 1996; Shimoda-Matsubayashi et al., 1996; Van Landeghem et al., 1999). This amino acid substitution may lead to absolute or relative deficiencies of critical enzymes within specific cellular compartments (Rosenblum et al., 1996). It has been reported that this single nucleotide polymorphism may be associated with an increased cancer risk.
Some studies have suggested that a consistently diminished amount of MnSOD can be found in cancer patients (Oberley and Buettner, 1979).

Single nucleotide polymorphisms of MnSOD are associated with an increased risk of breast cancer (Li et al., 1995; Ambrosone et al., 1999; Zhang et al., 1999; Mitrinen et al., 2001) malignant melanoma (Church et al., 1993), oral squamous cell carcinoma (Liu et al., 1997), oesophageal cancer (Toh et al., 2000), lung fibroblasts (Yan et al., 1996) glioma (Zhong et al., 1997) and prostate cancer (Woodson et al., 2003). In 2003, a US study did not corroborate an association between the Ala-9Val polymorphism and the development of breast cancer (Egan et al., 2003). In 2004, another study found that the Ala allele of MnSOD may modify breast cancer risk among current smokers, but is not an independent risk factor for breast cancer (Tamimi et al., 2004). By contrast, individuals heterozygous or homozygous for the MnSOD Val allele were reported to have a significantly elevated risk for lung cancer (Wang et al., 2001). Moreover, in the same study, a gene dose–response effect with an increasing cancer risk for each additional Val allele was discovered.

Moreover, MnSOD expression has been reported to be significantly reduced in patients with Barrett’s oesophagus with high-grade dysplasia and oesophageal adenocarcinoma (Hermann et al., 2005). In squamous cell carcinoma patients, Hu and colleagues found an overexpression of an MnSOD isoform and that its up-regulation in oesophageal cancer cells was associated with apoptosis resistance (Hu et al., 2007). However, this cannot be explained by MnSOD polymorphisms, as these changes are not induced by direct DNA damage.

To our knowledge, there has been no report so far investigating the relationship between the polymorphism of the MnSOD gene and the risk of oesophageal cancer. Since the cancer risk itself is strongly associated with the main risk factors such as alcohol and smoking, we performed a study comparing the MnSOD allele frequency in an oesophageal cancer cohort (both adeno- and squamous cell carcinoma) to a population-based study group of healthy blood donors as well as to a cohort of heavy drinkers.

### MATERIALS AND METHODS

#### Study population

All participants in this study were of Caucasian origin and were seeking treatment in the University Hospital Schleswig-Holstein, Campus Lübeck (Germany). All subjects were enrolled into the study between January 1998 and January 2006.

The cancer group (n = 170) consisted of 61 patients with adenocarcinoma as well as 109 patients with squamous cell carcinoma. All of these patients had undergone upper gastrointestinal tract endoscopy, had a histologically proven cancer in the oesophagus and were enrolled prior to cancer therapy. The histology was determined by an experienced pathologist of the University Clinic of Lübeck. As controls, 400 healthy blood donors were enrolled. In order to investigate the possible confounding effect of alcohol and smoking, a group of 160 heavy drinkers were included. These were patients seeking treatment for alcohol withdrawal.

Exclusion criteria for these two groups were history of any malignant tumour, clinical and laboratory signs of alcoholic liver cirrhosis and/or pancreatitis. The groups were matched for gender. For patients’ characteristics, see Table 1.

Each participant independently completed a structured questionnaire concerning drinking and smoking habits. The frequencies of both alcohol and tobacco consumption and the usual amounts were recorded.

#### Genotyping

Genomic DNA was isolated from whole blood using QIAGEN Genomic DNA kits. PCR-based restriction fragment length polymorphism (RFLP) assays were performed. Twenty pools of primers (5'-ACC AGC AGG CAG CTG GCG CCG G-3' and 5GCG TTG ATG TGA GTG TCC AG-3') reacted with 1 mM MgCl$_2$ and 1.25 U of Taq DNA polymerase in 50 μl systems. The PCR reaction was conducted according to Ambrosone’s protocol (Ambrosone et al., 1999). The 107 bp PCR product was incubated in 37°C with Ngo MIV (New England Biolabs) at 37°C for 4 h. The MnSOD A allele was cut into two fragments of 89 bp and 18 bp respectively, whereas the V allele remained intact and consisted of 107 bp. Generated fragments were separated on a 4% Metaphor agarose gel and subsequently stained with ethidium bromide. In order to assure the reliability of genotyping, the results of genotyping were evaluated by two examiners (L.S. and N.H). No interobserver variability was observed. Ten percent of the samples were analysed as blinded repeats, without any discordance.

#### Statistics

The main aim of this study was to investigate the effect of the MnSOD genotype on the development of oesophageal cancer. For this, in the first step, logistic regression models were built to predict the case–control status from age, sex, alcohol intake, number of cigarettes and all two-way interactions. To assess an independent effect of MnSOD genotypes on the case–control status, in the second step, MnSOD was added to the resulting

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**Table 1. Patients characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Heavy drinkers</th>
<th>Blood donors</th>
<th>All</th>
<th>Adenocarcinoma</th>
<th>Squamous cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>160</td>
<td>400</td>
<td>170</td>
<td>61</td>
<td>109</td>
</tr>
<tr>
<td>Age</td>
<td>50.5 ± 11.3</td>
<td>46.3 ± 8.8</td>
<td>63.6 ± 10.1</td>
<td>66.2 ± 10.7</td>
<td>62.1 ± 9.4</td>
</tr>
<tr>
<td>Gender (male/female in %)</td>
<td>123/37</td>
<td>312/88</td>
<td>132/38</td>
<td>50/11</td>
<td>81/28</td>
</tr>
<tr>
<td>Alcohol (g/day)</td>
<td>181.2 ± 135.9</td>
<td>6.5 ± 9.6</td>
<td>89.9 ± 93.8</td>
<td>59.2 ± 82.7</td>
<td>107.5 ± 95.6</td>
</tr>
<tr>
<td>Tobacco (cigarettes/day)</td>
<td>15.9 ± 15.1</td>
<td>4.3 ± 4.4</td>
<td>15.7 ± 14.6</td>
<td>11.8 ± 12.9</td>
<td>17.9 ± 15.1</td>
</tr>
</tbody>
</table>

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**Genetic Systems.**
MnSOD Polymorphisms in Oesophageal Cancer

Table 2. Comparison of oesophageal cancer patients with heavy drinkers

<table>
<thead>
<tr>
<th></th>
<th>Heavy drinker N (%)</th>
<th>Oesophageal cancer N (%)</th>
<th>OR 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn-SOD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val/Val</td>
<td>43 (26.9)</td>
<td>35 (20.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val/Ala</td>
<td>80 (50.0)</td>
<td>88 (51.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala/Ala</td>
<td>37 (23.1)</td>
<td>47 (27.7)</td>
<td>0.926</td>
<td>0.630–1.361</td>
</tr>
</tbody>
</table>

Patients with oesophageal cancer were not separated by histology in this table. For genotypes in adeno- and squamous cell carcinoma, see Table 3.

OR = odds ratio from multivariate analysis controlling for the logistic regression model considering the variables age and alcohol.

95% CI = 95% confidence interval for odds ratio.

Ethics

The study protocol was approved by the local ethics committee of the University of Lübeck.

RESULTS

The genotyping success was 100%. Comparing oesophageal cancer cases to heavy drinkers as well to healthy blood donors, we observed a more frequent Ala/Ala genotype among cancer patients (27.7% in cancer patients versus 23.1% in patients with heavy alcohol intake versus 12.5% in the group of healthy blood donors). In detail, the Ala/Val genotype was 51.8% in cancer patients versus 50% in patients with heavy alcohol intake versus 52.8% in the group of healthy blood donors and the Val/Val genotype was 20.6% in cancer patients versus 26.9% in patients with heavy alcohol intake versus 34.6% in the group of healthy blood donors. However, these differences were not significant in multivariate analyses controlling for age, alcohol and cigarettes (Tables 2 and 3). Analysing the oesophageal cancer patients separately for adenocarcinoma and ESCC according to histology, similar results were obtained (see Table 3).

Subjects with an Ala/Ala genotype (81.3 g/day) had a higher alcohol intake than those with Val/Ala (63.9 g/day) or Val/Val (53.8 g/day) genotype (exploratory P < 0.00001). Similar results were obtained with regard to tobacco consumption, with a higher proportion of smokers with Ala/Ala genotype (11.2 cigarettes/day) than those with Val/Ala (9.3 cigarettes/day) or Val/Val (8.8 cigarettes/day) genotype (exploratory P = 0.041, see also Table 4). The effect of age, alcohol and tobacco was similar in both subgroups as compared to the general cancer group. However, the statistical power for subgroup analysis was too low.

DISCUSSION

Alcohol, together with tobacco smoke, is the main cause for upper GI tract cancer in industrialized countries (Homann, 2001). One possible mechanism of alcohol-induced carcinogenesis is the metabolism of alcohol to acetaldehyde and NADH, which act as substrates for the formation of reactive oxidative species (Wright and Repine, 1999).

In patients with oesophageal adenocarcinoma, gastrooesophageal reflux disease appears to cause cancer through an inflammatory pathway driven by free oxygen radicals, as some groups found extensive oxidative DNA damage in an animal model of reflux disease (Sihvo et al., 2002). Manganese superoxide dismutase expression has been reported to be significantly reduced in patients with Barret’s oesophagus with high-grade dysplasia and oesophageal adenocarcinoma (Hermann et al., 2005; Yan et al., 2007). In squamous cell carcinoma patients, Hu and colleagues showed MnSOD overexpression and

Table 3. Comparison of oesophageal cancer patients with blood donors

<table>
<thead>
<tr>
<th></th>
<th>Blood donors N (%)</th>
<th>Oesophageal carcinoma N (%)</th>
<th>Adenocarcinoma N (%)</th>
<th>SCC N (%)</th>
<th>Multivariate analysis OR 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn-SOD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val/Val</td>
<td>139 (34.8)</td>
<td>35 (20.6)</td>
<td>10 (16.4)</td>
<td>25 (22.9)</td>
<td>1.026&lt;0.516–2.039</td>
<td>0.9426</td>
</tr>
<tr>
<td>Val/Ala</td>
<td>211 (52.8)</td>
<td>88 (51.8)</td>
<td>33 (54.1)</td>
<td>55 (50.5)</td>
<td>1.584&lt;0.715–3.510</td>
<td>0.2567</td>
</tr>
<tr>
<td>Ala/Ala</td>
<td>50 (12.5)</td>
<td>47 (27.7)</td>
<td>18 (29.5)</td>
<td>29 (26.6)</td>
<td>0.675&lt;0.228–1.994</td>
<td>0.4769</td>
</tr>
</tbody>
</table>

OR = odds ratio from multivariate analysis controlling for the logistic regression model considering the variables age, alcohol and cigarettes.

95% CI = 95% confidence interval for odds ratio.

<Values as compared for the oesophageal carcinoma group versus blood donors.

bValues as compared for the subgroup of patients with adenocarcinoma versus blood donors.

cValues as compared for the subgroup of patients with squamous cell carcinoma versus blood donors.

Table 4. MnSOD genotype and alcohol and tobacco consumption

<table>
<thead>
<tr>
<th></th>
<th>Val/Val</th>
<th>Val/Ala</th>
<th>Ala/Ala</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%)</td>
<td>217 (29.7)</td>
<td>379 (51.9)</td>
<td>134 (18.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Alcohol consumption (g/day)</td>
<td>53.8 (99.1)</td>
<td>63.9 (105.9)</td>
<td>81.3 (103.6)</td>
<td>0.041</td>
</tr>
<tr>
<td>Tobacco consumption (cigarettes/day)</td>
<td>8.8 (13.2)</td>
<td>9.3 (12.8)</td>
<td>11.2 (12.7)</td>
<td>0.041</td>
</tr>
</tbody>
</table>

P value = exploratory two-side P value from the Kruskal–Wallis test.
that MnSOD up-regulation in oesophageal cancer cells was associated with apoptosis resistance (Hu et al., 2007).

Thus, we hypothesized that the MnSOD Ala/Ala phenotype allele could be related to oesophageal cancer risk by having an altered capacity to reduce oxidative stress in both adenocarcinoma. In previous studies by other researchers, genotype MnSOD Ala/Ala was shown to increase the mitochondrial enzyme activity of MnSOD by 40% compared with the other two genotypes (Sutton et al., 2003). To our knowledge, this study is the first to examine the association between the MnSOD genetic polymorphism and oesophageal cancer risk. We observed that the MnSOD Ala/Ala phenotype predominated in the oesophageal cancer group compared to the control group, but this predominance was not an independent risk factor in multivariate analyses. Moreover, CIs for the ORs showed that large and meaningful effects can be excluded with high probability.

In our study, the only polymorphism studied was the T to C substitution, which was found leading to an amino acid substitution at −9 position in the signal peptide from valine (Val) to alanine (Ala). Other polymorphisms including 158T and differences in the enhancer region are other interesting candidates effecting malignant potential (Zhang et al., 1999; Kinningham et al., 2001).

The role of MnSOD in oxidative damage has been investigated in vivo and in vitro. Animal research showed increased MnSOD levels after exposure to a subacute ethanol intoxication (Pani et al., 2004). Chronic ethanol feeding caused an up-regulation of the enzyme at the mRNA level, with a good correlation between the transcript and the enzyme activity during the first 2 weeks of treatment. After 20 days, the mRNA level dropped to normal, whereas the activity still remained high (Koch et al., 1994). Gilks et al. found that cigarette smoke, like other forms of oxidant attack, increases the expression of MnSOD (Gilks et al., 1998). For both chronic and acute alcoholics, the increase in MnSOD activity may play an important role in the regulation of mitochondrial susceptibility to ethanol-induced oxidative stress (Kanbagli et al., 2002). In this study, due to many more cases of smokers and drinkers in MnSOD Ala/Ala genotypes, this polymorphism maybe antagonistic to the up-regulation of MnSOD by alcohol and smoking consumption. However, as we did not measure enzyme activity, this is only hypothesis. Thus, future studies should focus on correlation studies of polymorphisms and MnSOD expression to clear up this point. However, our study is the first to imply that polymorphisms in MnSOD may regulate alcohol and tobacco consumption. This leads to the interesting finding that some articles draw contradictory conclusions of either small or non-overall association of the Val-9 Ala MnSOD polymorphism with the development of breast cancer (Egan et al., 2003; Milikan et al., 2004; Tamimi et al., 2004).

Tamimi et al. found that the Ala allele of MnSOD may modify breast cancer risk among current smokers but is not an independent risk factor for breast cancer. In their study, there was a statistically significant interaction between MnSOD genotype and cigarette smoking status. This is very interesting, as alcohol and tobacco consumers often show a concomitant abuse and, moreover, alcohol is also a strong risk factor for breast cancer (Pöschl and Seitz, 2004). Thus, as the co-effect of alcohol abuse in some of the breast cancer studies was not thoroughly investigated, our finding of alcohol as a strong confounder could in part explain the different results.

However, the association between alcohol intake and MnSOD polymorphism is unclear, and Larosche and colleagues could show in a nice animal model that prolonged ethanol administration depletes mitochondrial DNA in MnSOD-overexpressing transgenic mice but not in their wild-type littermates (Larosche et al., 2009). One could speculate that Ala/Ala genotypes may lead to lower levels of MnSOD and individuals could drink more alcohol without any deleterious effects. However, future studies are warranted. Moreover, to prove the theory of a causal link between alcohol intake and MnSOD genotyped analysis of groups with heavy drinkers with limited tobacco smoke versus heavy smokers and minimal drinkers would be warranted.

The potential role of MnSOD and its polymorphisms in Barrett’s oesophagus is controversial. As Hermann et al. could show a reduced MnSOD expression in patients with Barrett’s oesophagus with high-grade dysplasia and oesophageal adenocarcinoma, Murphy et al. showed that failed MnSOD genes were associated with Barrett’s oesophagus (Hermann et al., 2005; Murphy et al., 2007).

In conclusion, our study failed to demonstrate an association between MnSOD genotypes and oesophageal carcinoma. However, our results indicated interesting associations between MnSOD genotype and alcohol and tobacco use, suggesting a complex gene-to-phenotype interaction between these risk factors for cancer development and oxidative stress repair.

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REFERENCES


