Increased activity of superoxide dismutase in advanced stages of head and neck squamous cell carcinoma with locoregional metastases

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The aim of the study was to investigate relationship between activity of superoxide dismutase (SOD), malondialdehyde (MDA) and tumor necrosis factor α (TNFα) and between Ala-9Val polymorphism in the gene encoding MnSOD (*SOD2*) and the initial stage and prognosis of the head and neck squamous cell carcinoma (HNSCC).

Prospective study cohort comprised 88 patients who underwent surgical treatment for the diagnosis of HNSCC (53 patients were diagnosed with locoregional metastatic spread (N+) at the time of diagnosis). After the initial surgery subjects were followed for the subsequent period of 26 months during which 14 manifested relapse. Genotypes were detected by the PCR-based methodology. Activity of p-SOD, ery-SOD and TNFα were determined by ELISA, and the concentration of MDA by high performance liquid chromatography.

Genotype and allele frequencies of the Ala-9Val differed neither between groups defined according to the stage of primary disease (TNM), nor between relapse vs. remission groups after the follow-up ($p>0.05$). Activity of p-SOD was significantly higher in T3/4 stage compared to T1/2 ($p=0.01$) and was also higher in N+ compared to N0 patients ($p=0.002$). Carriers of the Ala/Ala genotype had higher p-SOD activity ($p=0.04$). There was no significant difference in DFI between *SOD2* genotype groups ($p>0.05$), however, the Ala/Ala group exhibited the shortest median DFI.

In conclusion, our results suggest that increased p-SOD at the time of the initial treatment for HNSCC is connected with greater extent and nodal metastatic spread of the initial disease and with an earlier relapse of the disease. Progression of the disease might be further modified by the presence of Ala/Ala genotype of the *SOD2*. Activity of p-SOD could thus offer diagnostic as well as prognostic value.

Keywords: antioxidative enzymes, head and neck cancer, metastasis, oxidative stress, polymorphism, superoxide dismutase

Clinical and experimental studies have provided sufficient background supporting an important role of oxidative stress in cancer development [1, 2]. Oxidative stress, a situation of profound imbalance between the oxidant and antioxidant potential of cells, results either from an overproduction of reactive oxygen species (ROS), insufficient detoxification of ROS by antioxidative enzymes or a combination of both. ROS is a term usually reserved to describe a group of highly reactive forms of oxygen carrying one or more unpaired electrons. They include superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, etc. The source of ROS evoking oxidative stress is either exogenous or endogenous. The most important exogenous oxidants are ozone and cigarette smoke. Endogenously, numerous inflammatory cells, e.g. eosinophils, alveolar macrophages, etc., release ROS. However, mitochondria, more precisely protein complexes of the mitochondrial respiratory chain, are a dominant source of endogenous ROS. Surplus electrons, unused for generation of the proton gradient, can interact with oxygen to produce superoxides. If these are not neutralized rapidly enough by the action of antioxidative enzymes (superoxide dismutases, catalase etc.) and non-enzymatic antioxidants, the intracellular structures, i.e. proteins, lipids and DNA, are damaged by oxidation [3]. The extent of oxidative damage of the cellular membrane phospholipid bilayer by lipid peroxidation can be assessed by

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Abbreviations – AOE – antioxidative enzymes, CR – complete remission, CUP – cancer of unknown primary, DFI – disease free interval, HNSCC – head and neck squamous cell carcinoma, IL-1 – interleukin 1, ROS – reactive oxygen species, SOD2 – manganese superoxide dismutase, p-SOD – plasmatic superoxide dismutase, ery-SOD – superoxide dismutase in erythrocytes, TNFα – tumour necrosis factor alpha

malondialdehyde (MDA) – a product of lipid peroxidation and a useful circulating biomarker of oxidative stress. MDA can further react with macromolecules incl. DNA, thus, possibly contributing to mutagenesis and cancerogenesis [4].

Activation of redox-sensitive transcription factors (e.g. NFκB or AP-1) represents another consequence of increased oxidative stress. This alters cellular phenotype due to the gene expression of pro-inflammatory mediators, e.g. tumour necrosis factor α (TNFα), interleukins etc., that further multiplies intracellular damage. TNFα is a proinflammatory cytokine leading to the generation of ROS in cells.

Manganese superoxide dismutase (MnSOD) is one of the key enzymes responsible for a detoxification of ROS in the mitochondria present in almost all aerobic organisms. Apart from the mitochondrial form, two other forms containing copper and zinc are localised in cytoplasm (SOD1) and extracellularly (SOD3) [5]. Individual differences in the activities of SOD2 (as a result of genetic variability) are considered as potentially important factors in the pro-oxidative settings. The gene encoding MnSOD (*SOD2*, MIM 147460, chromosome 6q25.3) is composed of five exons and four introns. Matsubayashi described the C → T substitution in nucleotide 47, resulting in the Ala (GCT) → Val (GTT) alteration of codon 16, and change at the -9 position (Ala-9Val) of the mitochondrial targeting sequence. The -9Val substitution alters the secondary alpha-helix structure of the protein, and misdirects intracellular trafficking of MnSOD into the mitochondria [5]. The mutant protein has approx. 30 to 40% lower activity and increases susceptibility to oxidative stress.

Several authors studied the relationship between oxidative burden and cancer incidence and prognosis over the past several years. Oxidative stress is related to cancer in multiple ways. Firstly, oxidative stress can mediate oxidative damage to DNA and thereby initially promote carcinogenesis. Secondly, many malignant cells have an abnormal ability to cope with oxidative stress due to alterations in their antioxidant properties. One of the cancers with the strongest link to oxidative damage and oxidative stress is head and neck squamous cell carcinoma (HNSCC), since tobacco and alcohol, as sources of massive quantities of ROS, are clearly identified as etiologic factors of these malignancies [6]. Up to now, no predictive marker of metastatic dissemination of HNSCC to neck lymph nodes has been identified; therefore, numerous patients without locoregional metastases undergo elective neck lymph node dissection with all potential negative side effects and complications. A reliable prognostic marker of metastatic spread could save up to 60-80% of these patients from neck surgery, since Schroder showed that only 20-40% of histologic samples from elective neck dissections were positive for squamous cell carcinoma metastases [7]. We therefore hypothesised that identification of predictive markers could represent a very important tool for determining the appropriate extent of surgical treatment of HNSCC patients who are N0 at the time of surgery.

The primary aim of the study was to investigate relationships between Ala-9Val substitution in the *SOD2* gene, circulating levels of selected markers of oxidative stress (activity of the erythrocytes and plasma SOD, concentration of MDA and TNFα) and prognosis of HNSCC (i.e. prognostic value). The secondary aim was to ascertain the eventual relationship between the above mentioned parameters and the extent of primary disease at the time of diagnosis (i.e. diagnostic value).

Patients and Methods

Study subjects. Prospective study cohort comprised a total 88 patients (78 men, 10 women, mean age 59.1±8.8 yrs, range 33 to 83 yrs) who underwent surgical treatment for HNSCC at The Clinic of Otorinolaryngology and Head & Surgery of Masaryk University in Brno, Czech Republic between July 2004 and December 2005. Diagnosis of HNSCC in primary tumor was confirmed in 86 cases, 2 patients were treated for locoregional metastases of squamous cell carcinoma in the neck lymph nodes with an unknown primary tumor. Staging (according to the TNM classification) was as follows: 6 patients as T1, 34 as T2, 23 as T3 and 23 as T4; 35 patients had no lymph node metastatic spread (N0), 17 patients were N1, 34 patients were classified as N2 and 2 as N3. Histopathologic examination characterized 17 tumours as well differentiated squamous cell carcinoma, 45 as moderately differentiated and 20 as poorly differentiated; differentiation was not possible in the 6 remaining samples. The TNM stage was classified according to 1997 American Joint Committee on Cancer system.

Genetic analysis. Genomic DNA was isolated from peripheral blood leukocytes by a standard method using proteinase K. The Ala-9Val single nucleotide polymorphism in the signal peptide of *SOD2* gene was determined as previously described [8]. Briefly, a 249bp fragment of the *SOD2* signal sequence was amplified using the following primers: 5'-AGCCCAGCCGTGCGTAGAC-3' and 5'-TACTTCTCCTCGGTGACG-3'. PCR products were digested using the BsaWI restriction endonuclease (New England BioLabs), digestion products (249bp for the Ala-9 allele, 87 and 162bp for the Val-9 allele) were separated by electrophoresis in 2% agarose gel and visualized under UV light.

Biochemical assays. Activity of SOD was determined in plasma (p-SOD) and in erythrocytes (ery-SOD) using commercial assay kits according to manufacturer recommendations (Cayman Chemical Co., Ann Arbor, MI). MDA) as a marker of lipoperoxidation was assessed in plasma using liquid chromatography as described previously [9]. Plasma TNFα was determined by ultrasensitive ELISA (BioSource, Camarillo, CA).

Statistical analysis. Allele frequencies were calculated from the observed numbers of genotypes. Deviation of genotype distribution from Hardy-Weinberg equilibrium was tested by

χ^2-test. Comparisons of quantitative parameters (activities of ery-SOD, p-SOD, MDA and TNFα) corresponding to particular genotype groups of the Ala-9Val polymorphism were performed by Kruskal-Wallis ANOVA. Comparisons of groups were performed by Mann-Whitney U-tests. Spearman correlation coefficients were used to assess correlation between parameters. Kaplan-Meier survival analysis was used to estimate disease-free interval (DFI). $P< 0.05$ was considered statistically significant. All the analyses were performed with STATISTICA v. 7.1 by StatSoft, Inc. (USA).

Results

A. Inception cohort – relationship between parameters studied and primary disease

Genotype distribution of the Ala-9Val polymorphisms in the SOD2 gene. Genotype distribution for the Ala-9Val polymorphism did not deviate from Hardy-Weinberg equilibrium (P>0.05). Allele frequencies were 55.3% for alanine and 44.7% for valine. Table 1 shows genotype distribution in the particular subgroups according to the TNM stage of primary disease. There were no significant differences neither between groups defined according to the T nor N stage ($p>0.05$, χ^2-test).

Genotypes vs. circulating levels of p-SOD, ery-SOD, MDA and TNFα. The mean activities of SOD were 12,132 ± 4,799 U/g in plasma and 4,152 ± 840 U/g in erythrocytes, MDA was 2.10 ± 0.62 nmol/g of protein and TNFα was 4.38 ± 2.08 ng/L. Activity of ery-SOD negatively correlated with plasma level of MDA ($p<0.05$, Spearman).

Activities of p-SOD, ery-SOD, MDA and TNFα were compared among patients with different TNM stages. Activity of p-SOD was significantly higher in the pooled T3/4 group compared to T1/2 (p=0.01, Mann-Whitney). Furthermore, activity of p-SOD was higher in the pooled N+ group compared to N0 patients (see Figure 1) (p=0.002, Mann-Whitney). Other parameters studied did not reveal significant differences between groups.

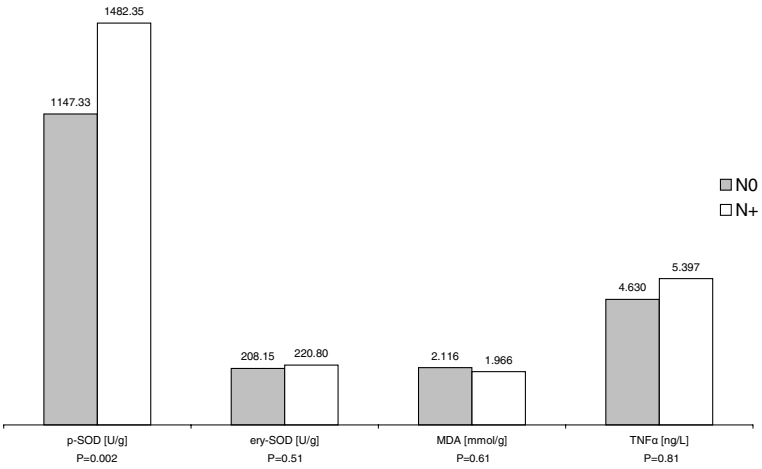

Figure 1. Activities of p-SOD and ery-SOD, respectively levels of MDA and TNFα, in patients with/without metastatic spread to neck lymph nodes.

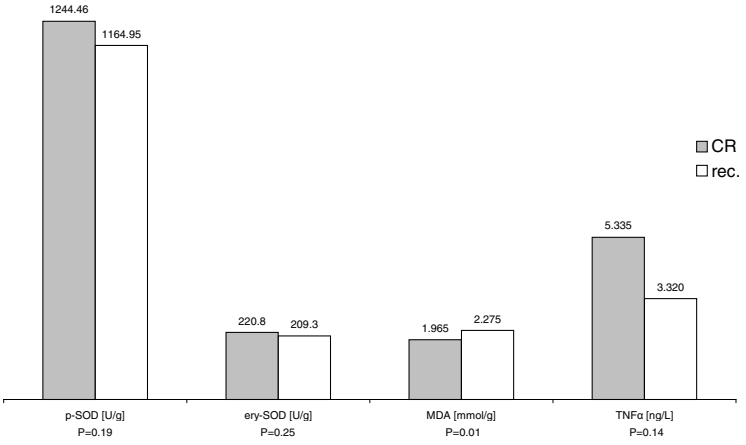

Figure 2. Activities of p-SOD, ery-SOD, levels of MDA and TNFα in patients remaining in the complete remission (CR) and those with relapse (rec.).

Activities of p-SOD corresponding to the particular genotypes of the Ala-9Val polymorphism differed significantly (p=0.02, Kruskal-Wallis). Carriers of the Ala/Ala genotype exhibited the highest p-SOD activity (Ala/Ala =1655.99 ± 625.72 U/g, Ala/Val = 1300.84 ± 535.43 U/g, Val/Val = 1465.45 ± 485.08 U/g). There were no significant associations between genotype and other measured parameters.

B. Prospective follow-up – relationship between parameters studied and progression of disease

Out of the total number of 88 patients included in this study, 4 did not reach complete remission (CR) and were omitted from subsequent analyses. A subset of 14 subjects (out of remaining 84) manifested disease relapse during the 26 month-period following the initial surgery while 70 patients stayed in CR.

Comparison of CR vs. relapse group did not revealed significant difference in allele frequencies of the *SOD2* SNP ($p>0.05$, χ^2 test, see Table 2). Plasma level of MDA was significantly higher in the relapse group than in CR group (p=0.01, Mann-Whitney, Figure 2). Other parameters did not differ between these two groups of patients.

Survival analysis of disease free interval. By means of Kaplan-Meier survival analysis we analysed DFI in groups defined according to the Ala-9Val genotypes. Patients remaining in the CR during the 26 month follow-up were classified as "censored responses", those with relapse as "complete responses". Median DFIs were 16.7 months in the Ala/Ala, 18.7 months in the Ala/Val group, while neither median nor 25-percentile was reached in the Val/Val group. Comparison of

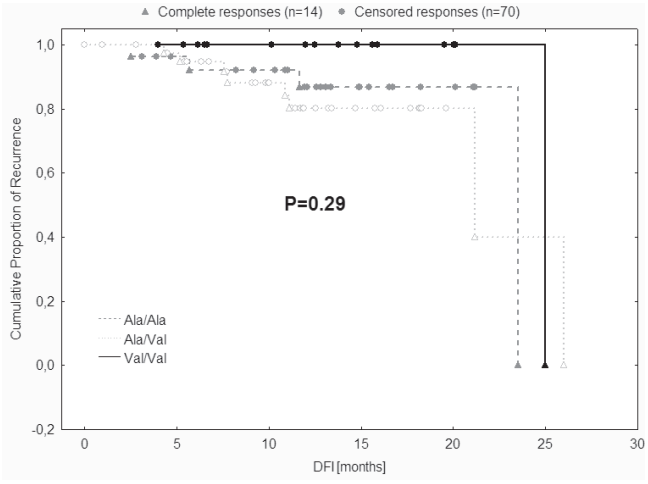

Figure 3. Kaplan-Meier survival curve showing DFI in different genotype groups of the *SOD2* Ala-9Val polymorphism.

the three groups did not revealed statistical significance (P>0.05), similarly, post-hoc comparison of pairs of groups showed no significant differences (all P>0.05, log-rank test). Nevertheless, a tendency towards longer DFI (thus consequently better prognosis) in the Val/Val group was apparent (see Figure 3).

Discussion

Using prospective cohort study design we investigated association of the functional polymorphism in the *SOD2* gene – Ala-9Val – with both stage of the primary disease – HNSCC – at the time of diagnosis (i.e. diagnostic value), and with the progression of disease from the CR during the 26 month follow-up (i.e. prognostic value). Furthermore, we assessed similar associations for the panel of selected circulating markers of oxidative stress – erythrocyte and plasma activity of SOD, concentration of MDA and TNFα. Our findings can be summarized as follows: (1) activity of plasma SOD was significantly higher in patients with advanced stages of HNSCC (T3/4) and those with metastatic spread into the neck lymph nodes (N+), (2) level of lipid peroxidation (MDA) was significantly elevated in patients who later manifested recurrence of cancer, (3) plasma SOD significantly differed between genotype groups of the *SOD2* polymorphism with carriers of the Ala/Ala genotype having the highest plasma SOD levels, finally, (4) carriers of the Ala/Ala genotype had the shortest median of the disease-free interval, which indirectly supports the role of genetic variability in the *SOD2* gene as a prognostic marker, while direct association of the allele or genotypes of the SNP studied was not found.

Table 1. Allele and genetic variant distribution of SOD2 Ala-9Val polymorphism in the particular subgroups according to the TNM classification.

	CUP	T1	T2	T3	T4	N0	N1	N2	N3
Ala/Ala	0	2	11	6	8	6	8	11	2
Ala/Val	1	2	15	13	9	19	6	15	0
Val/Val	1	2	7	3	5	8	3	7	0
	p=NS					p=NS			

Abbreviations: CUP – cancer of unknown primary. Note: genotyping was not complete, genotypes were missing in 3 subjects due to lack of template or consistent PCR drop-out.

Increased activity of AOE in patients with advanced cancer disease has already been published by Sabitha and Jaloszynski [2, 4]. Szuster-Ciesielska reported increased production of ROS correlating with the tumor stage in the laryngeal cancer [10]. Our findings of increased activity of p-SOD in the advanced T stages and N+ patients are in concert with both former studies and support the role of oxidative stress in the tumor pathogenesis.

The association between p-SOD activity and the metastatic spreading into locoregional lymph nodes in HNSCC certainly deserves further attention. If such relationship would be proven in the larger study group (currently ongoing), the plasma activity of superoxide dismutase could become one of the indication factors for the eventual neck dissections in N0 patients. Those with low p-SOD activity could be potentially spared from the surgical dissection of the neck lymph nodes. Similarly, increased plasma levels of MDA in patients who manifested subsequent relapse later during the follow-up could potentially offer prognostic potential, although there are no other published data in the literature supporting this hypothesis.

Genetic variant studied – Ala-9Val polymorphism in *SOD2* gene – did not exhibit direct relationship with neither extent of initial disease nor its prognosis. However, when investigating temporal pattern of disease relapse (DFI) by survival

Table 2. Comparison of allele and genotype frequencies in the group of patients remaining in the CR and those manifesting relapse during 26 months follow-up.

Patient status	n	Ala/Ala	Ala/Val	Val/Val	Ala	Val
all	n=88	27 (31.8%)	40 (47.1%)	18 (21.1%)	94 (55.3%)	76 (44.7%)
reached CR	n=84	27 (33.3%)	39 (48.2%)	15 (18.5%)	93 (57.4%)	69 (42.6%)
(a) remained in CR	n=70	23 (33.8%)	31 (45.6%)	14 (20.6%)	77 (56.6%)	59 (43.4%)
(b) relapse	n=14	4 (30.8%)	8 (61.5%)	1 (7.7%)	16 (61.5%)	10 (38.5%)

analysis there was an obvious tendency towards shorter DFI in Ala/Ala carriers, the very genotype significantly associated with the highest activity of p-SOD.

In conclusion, our study suggests that advanced disease stages in patients with HNSCC with locoregional metastases are accompanied by an increased oxidative stress. Increased concentration of plasma oxidative stress markers – SOD activity and lipoperoxidation product (MDA) – can serve as handy diagnostic or prognostic tools, respectively. Detail elucidation of the mutual relationships between the plasma activity of SOD, genetic variability in the SOD2 gene and disease progression is worth further study.

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