Elevated Malondialdehyde Correlates with the Extent of Primary Tumor and Predicts Poor Prognosis of Oropharyngeal Cancer

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Abstract. The aim of the study was to investigate the relationship between plasma levels of malondialdehyde (MDA), a routinely used marker of oxidative stress, and squamous cell carcinoma of the oral cavity and oropharynx (OSCC). The prospective cohort study comprised a total of 67 patients who underwent surgery for OSCC. MDA was assessed using high performance liquid chromatography. The MDA levels in the pooled T1-2 patients were lower than in the patients with T3-4 tumors. A negative correlation of MDA and tumor grade was shown. Seventeen patients who manifested recurrence during the 49.6 months follow-up had significantly increased MDA compared to those staying in complete remission. Kaplan-Meier analysis revealed that the median disease-free interval and overall survival in the group with MDA>median was 19.3 and 22.5 months respectively, in contrast to 31.5 and 31.6 months respectively, in patients with MDA≤median. The prognostic value and low cost of MDA measurement could make it a versatile and useful prognostic tool for the identification of OSCC patients with a high risk of recurrence.

There is substantial evidence that oxidative stress participates in carcinogenesis (1-4). Oxidative stress, defined as an imbalance between reactive oxygen species (ROS) production and removal, results from the overproduction of ROS, decreased antioxidant defence or a combination of both. ROS-induced damage of macromolecules can lead to changes of their structure and, consequently, function. Oxidative damage of membrane phospholipids is called lipid peroxidation with malondialdehyde (MDA), an end-product of lipid peroxidation, being a widely used marker of cell exposure to oxidative stress (2-6). In addition, MDA is suggested to act as a tumor promoter and co-carcinogenic agent due to its high cytotoxicity (2, 3).

Alterations in redox cell signalling produce changes that, synergistically with DNA alterations, induce deregulation of cell cycle control and cell proliferation (1). ROS have been implicated as second messengers involved in the activation of nuclear factor κB (NF-κB) directly or via cytokine production (2). Several authors have correlated the rate of ROS production with p53-mediated apoptosis. Furthermore, increased levels of ROS have been shown to induce p53 mutations (7, 8).

Relationships between ROS, uncontrolled cell proliferation, activation of NF-κB and overexpression of p53 point to the significance of oxidative stress in cancer development. Carcinogenesis is a complex multi-stage process, when the cell phenotype undergoes changes from healthy through precancerous to malignant. Initiation as the first stage of the “initiation-promotion-progression” model of carcinogenesis (9, 10) involves non-lethal DNA mutations in affected cell(s). The majority of DNA mutations very likely occur via the action of ROS. The promotion phase is characterized by the clonal expansion of initiated cells due to suppressed apoptosis. Many tumor promoters manifest inhibitory effects.
on antioxidative defence mechanisms, thus increasing the level of oxidative stress in the tumor cell. However, excessive levels of ROS are cytotoxic and lead to the induction of programmed cell death (11). Progression as the final stage of carcinogenesis involves irreversible changes from the benign to the malignant cell phenotype by the continuous accumulation of genetic alterations with oxidative stress probably playing a similar role as in the previous stages (9).

Squamous cell carcinoma (SCC) of the oral cavity and oropharynx (OSCC), the fifth most common cancer worldwide (4), represents a considerable health problem. Its treatment, both surgical and radiotherapy, is often mutilating and generally accompanied by serious and permanent side-effects (e.g. xerostomy, swallowing disorders, loss or substantial alteration of taste, etc.). Furthermore, OSCC is characterised by a high risk of recurrence. To date, a reliable tool for identifying patients who would benefit from radical surgery and those who could be spared from extensive resection is lacking.

Markers of oxidative stress seem very promising as molecular oncomarkers due to their significant role in the entire process of carcinogenesis. Head and neck oncology lacks verified and widely used oncomarkers. A literature survey revealed that only a very limited number of studies have dealt with lipid peroxidation, MDA in particular, in assessing the prognosis and treatment success of SCC. The identification of both diagnostic and prognostic oncomarkers in combination with traditional histopathological characteristics would surely lead to improved cancer therapy. In our previous work, perioperative superoxide dismutase activity in plasma was identified as an indicator of metastatic spread of SCC to neck lymph nodes (12). The current study aimed to investigate a potential prognostic role of another parameter of oxidative stress, MDA. The specific aims of the study were to find a possible relationship between plasma levels of MDA and oncological characteristics of OSCC and to clarify the prognostic role of MDA as a plasma-based molecular oncomarker.

**Patients and Methods**

**Study subjects.** This prospective cohort study comprised a total of 67 patients (57 men, 10 women, mean age 56.5±8.4 years, range 31 to 78 years) who underwent surgery as a primary treatment for OSCC at The Clinic of Otorhinolaryngology and Head and Surgery of Masaryk University in Brno, Czech Republic between July 2004 and November 2006. The median follow-up was 28.8 months (range 1.3 to 48.0 months). None of the patients had been treated with chemotherapy or radiotherapy for any oncological diagnosis prior to his/her inclusion in the study. The primary tumor was diagnosed in the tonsil or base of the tongue in 42 patients, in the anterior 2/3 of the tongue in 11 patients and in the floor of the mouth in 14 patients. Other localizations of primary tumor were not included in the study. Staging according to the TNM classification (13) was as follows: 6 patients were classified as T1, 24 as T2, 7 as T3 and 30 as T4; 16 patients had no lymph node metastatic spread (N0), 14 patients were N1, 35 patients were classified as N2 and 2 as N3. Histopathological examination characterized 12 tumors as well differentiated SCC, 33 as moderately differentiated and 17 as poorly differentiated; classification of tumor grade was not possible in the remaining 5 samples. Written informed consent was obtained from all the participants of the study. The study was conducted in accordance with the Helsinki declaration and was approved by the Ethical Committee of St. Anne’s University Hospital, Brno, Czech Republic.

**Biochemical assays.** Blood samples for the evaluation of MDA levels were taken on day 7 after surgical resection of the tumor. The plasma level of MDA was assessed after derivation by thiobarbituric acid (TBA) using high performance liquid chromatography with fluorescence detection as described previously by Khoschsorur et al. (14). Briefly, plasma aliquots were mixed with solutions of phosphoric acid (0.66 M) and TBA (0.21 M) and ultrapure water at volume ratio of 1:10:10:10. The mixture was heated at 95°C for 60 min. After cooling on ice, the mixture was neutralized by an equal volume of alkaline methanol containing 0.08 M NaOH and centrifuged. The supernatant was injected on to a LiChrosorb RP18 (150 mm × 4.6 mm, i.d.) column (Merck, Darmstadt, Germany) with a mobile phase containing 50 mM phosphate buffer, pH 6.8 and methanol (40:60, v/v). Separated MDA-TBA adducts were quantified fluorometrically with excitation at 527 nm and emission at 551 nm.

**Statistical analysis.** Comparisons of plasma MDA levels between groups (smokers vs. non-smokers, T stages, patients in complete remission vs. patients with recurrent disease) were performed by Mann-Whitney U-test. Spearman’s correlation coefficients were calculated to assess pair-wise correlations between variables. The effect of the MDA level on prognosis was analyzed using Kaplan–Meier survival analysis. Recurrence risks were compared by Fisher exact test. The Chi-square test was used for interval estimation of risk. P-value <0.05 was considered statistically significant. All the analyses were performed with STATISTICA v. 8.0 (Statsoft Inc., Tulsa, OK, USA).

**Results**

**MDA and primary disease.** Plasma MDA levels were compared between different TNM stages. The MDA plasma levels in the pooled T1-2 patients were significantly lower than in the T3-4 tumor patients (p=0.0009, Mann-Whitney, Figure 1). The plasma MDA was significantly correlated with T-stage (p=0.00001, r=0.49, Spearman). A negative correlation between MDA and tumor grade (p=0.03, r=–0.29, Spearman, Figure 2), was also shown.

**MDA and smoking.** Typically the majority of SCC patients are smokers, and all but nine of the patients included in the study were smokers. No difference in plasma MDA levels between the two (greatly disparate) subgroups was found (p>0.05, Mann-Whitney). The result was influenced by the low number of non-smoking individuals however.
MDA and disease relapse. Out of a total of 67 patients included in the study, 17 were lost to follow-up and were excluded from disease relapse analyses. Four of these patients who did not attend follow-up visits, but were alive because they visited our hospital for other than oncological reasons, were included in the survival analyses. A subset of 16 patients manifested recurrence during the 48 months follow-up period following the initial surgery while 34 stayed in complete remission. The levels of MDA were significantly increased in the relapse group in contrast to the complete remission group ($p=0.008$, Mann Whitney).

MDA level predicting risk of recurrence. Kaplan-Meier analysis was performed to ascertain the relationship between MDA and disease-free interval (DFI) and overall survival (OS). The distribution of MDA revealed a bi-apical pattern, supporting comparisons between the study sub-groups defined by the median. Kaplan-Meier survival curves were thus constructed for two groups of patients: those with MDA≤median and those with MDA>median. The patients remaining in complete remission or alive (for calculation of OS) during the 48 month follow-up were classified as “censored responses” and those with relapse or those who died as “complete responses”, respectively. The median DFI and OS in the group with MDA>median was 19.4 months and 22.5 months, respectively, in contrast to 31.5 months and 31.6 months, respectively, in the group with MDA≤median. The patients with MDA≤median had statistically significantly longer DFI ($p=0.01$, log rank test, Figure 3A) and longer OS ($p=0.04$, log-prank test, Figure 3B) compared to those with MDA>median.

The patients with MDA>median showed a recurrence rate of 48.0% (95% confidence interval (CI) 27.8-68.7%), i.e. 12 out of all 25 patients with MDA>median encountered...
disease relapse during the 48 month period following the initial surgery. In contrast, SCC recurred in the patients with MDA≤median only in 16.0% of the cases (95% CI 4.5-36.1%), i.e. 4 out of all 25 patients with MDA≤median. Therefore, the patients with high MDA perioperatively had a 3-times higher risk of a recurrence than the patients with low MDA (48.0% vs. 16.0%, Fisher exact test $p=0.01$). MDA revealed similar value in predicting a fatal prognosis with a 2-times higher risk in the high MDA patients compared the low MDA patients (59.3% vs. 29.6%, Fisher exact test $p=0.02$).

**Discussion**

The study findings can be summarized as follows: the patients with advanced head and neck cancer were exposed to higher oxidative stress when compared to early stage carcinomas; tumor grade showed good correlation with exposure to oxidative stress; no relationship between smoking and MDA was found; the perioperative level of oxidative stress was significantly elevated in the patients who manifested cancer recurrence later during the follow-up period and the patients with high plasma levels of MDA, i.e. higher than median, manifested a higher risk of a recurrence and death than the patients who perioperatively had low levels of oxidative stress.

The presented data supported our previous findings, in agreement with many other authors (15-17), that patients with advanced SCC and those with worse prognosis are perioperatively exposed to high levels of oxidative stress. Szuster-Ciesielska et al. proposed that tumor cells are the dominant producers of ROS and described the decrease of elevated levels of MDA after surgical removal of SCC of larynx (17). Hrizostov et al. demonstrated the normalization of preoperatively elevated plasma levels of MDA 10 to 20 days after surgery (18).

It is widely accepted that smoking influences the level of oxidative stress. Bloomer, studying young healthy individuals, described higher plasma MDA in smokers than in non-smokers (19). However, we found no reports of MDA levels in cancer patients with differing smoking habits. The present study revealed no differences in MDA level when smokers and non-smokers were compared but the low number of non-smokers in the study group, i.e. 9 out of 67 patients, limited the clinical impilcation.

With the exception of our earlier study of all primary localizations of SCC of the head and neck (12), we are not aware of any study focusing on a role for MDA in the prognostic assessment of head and neck cancer recurrence or survival. The present results supported our previous findings based on a relatively short follow-up, proposing MDA as a plasma prognostic oncomarker. The present study revealed that patients with low levels of ROS perioperatively, i.e. with low levels of MDA, were less likely to encounter recurrence and, consequently, were more likely to survive. This significant difference was shown not only by Kaplan-Meier analysis, but also in that the risk of recurrence between these two groups of patients differed by more than 3-times. Increased oxidative stress indicates a more aggressive carcinoma as a result of tumor suppressor inactivation (p53) and oncogen induction (ras) (20). The more aggressive the cancer, the worse the prognosis, with a higher recurrence rate. The stratification of patients according to the risk of recurrence could allow personalized oncological therapy. Consequently, high-risk patients could be targeted with more aggressive therapy and more extensive surgery of the primary tumor and neck lymph nodes (21) or, based on further sub-categorization, those with extremely poor prognosis might be spared from radical surgery (e.g. extensive disfiguring and mutilating resections) (22) altogether since they would not gain any advantage, increased quality of life or prolonged survival. A more detailed evaluation of correlation between the initial surgical technique and extent and prognosis is the aim of our ongoing investigations.

Finally, significant differences exist between patients according to their perioperative plasma MDA levels and the very low cost of plasma MDA measurement could make it a very versatile and easy-to-use prognostic marker. In accordance with other authors, we believe that MDA and several other parameters of oxidative stress could be established as oncomarkers for head and neck malignancies.

**Acknowledgements**

This project was supported by grant No. 10/II NR/9200-3 from The Internal Grant Agency of The Ministry of Health of the Czech Republic.

**References**

Salzman et al: MDA and Oropharyngeal Cancer

Received May 14, 2009
Revised August 5, 2009
Accepted September 2, 2009