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Oxidative stress in relation to adenosine deaminase, nitric oxide, nitric oxide synthase and xanthine oxidase in oral cavity cancer

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ABSTRACT

Objective. Inflammation and oxidative stress are considered as the main pathways in oral cavity carcinoma. The aim of this study was to evaluate the activity levels of adenosine deaminase (ADA), nitric oxide (NO), nitric oxide synthase (NOS), and xanthine oxidase (XO) in oral cavity carcinoma, to determine their potential roles in carcinogenesis with relation to oxidative stress. ***Methods.*** Seventeen patients with oral cavity cancer underwent surgery as the primary therapy, were consisted in the study. Resected oral cavity carcinoma tissues were compared with the adjacent tumor-free control tissues of the same patients; ADA, NO, NOS, XO activity levels were evaluated in terms of difference. ***Results.*** There is a significant increase of ADA activity in squamous cell cancer tissues, which indicates a difference between the normal and tumor tissues at enzyme levels (p<0.001). ***Conclusion.*** Elevated ADA activity might be an attempt to supress formation of the immunosupressed niche which promotes the onset of neoplasia and/or to inhibit tumor progression and metastasis.

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Keywords: Adenosine deaminase, nitric oxide, nitric oxide synthase, oral cancer, oxidative stress, xanthine oxidase

Introduction

Oral cavity cancer is the most common in the head and neck cancers and has a high morbidity and mortality. Certain risk factors for the development of oral cavity carcinoma including tobacco, alcohol, nutrition, viral infection, poor dentition are known; these factors explain 90-95% of the cases especially with squamous cell carcinoma [1].

Carcinogenesis studies emphasize that carcinogenic substances are needed to be metabolized by the enzyme systems. As regarding to this accepted hypothesis, these chemicals turn into the electrophilic metabolites (radicals) in the body and lead to the

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activation of oncogenes caused by DNA damage [2-4]. We think that these enzyme systems are more significant in relation to oxidative stress in cancer types, in which predisposing factors such as oral cavity cancers are frequent.

Nitric oxide (NO) is a free oxygen radical that has significant immune functions and produced from L-arginine by NO synthase (NOS). The specific effect of NO in carcinogenesis probably depends on its local concentration and it is suggested that NO facilitates tumor growth and increases angiogenesis. The effects of NO in carcinogenesis are; advancing tumoral vascularization and angiogenesis, facilitating tumoral cell adhesion to endothelial cells and, increasing the vascular permeability. All of these effects can facilitate the rapid growth of the primary tumor [5].

Adenosine deaminase (ADA) and xanthine oxidase (XO) are enzymes that participate in purine and DNA metabolism. These enzymes are needed for the turnover of nucleic acids in tissues. ADA irreversibly converts adenosine and deoxyadenosine to inosine and deoxyinosine. This pathway increases the formation of hypoxanthine and xanthine which is finally converted to uric acid by XO. These enzymes are shown as the central mechanism of FORs formation [6, 7].

The research question of our study is whether there is a difference between tumor and tumor-free tissue in terms of enzyme levels in relation to oxidative stress. The aim of this study was to evaluate the activity levels of ADA, NO, NOS and XO in tumor tissues of oral cavity, to determine their potential roles in carcinogenesis with relation to oxidative stress.

Methods

Patients

This study consisted of seventeen patients who underwent surgery for oral cavity cancer (median age of 58 years, range: 27 to 84 years) in Ear, Nose and Throat Departments of two tertiary referral centers. All patients were histologically diagnosed by incisional biopsy prior to surgical procedures. None of the patients had a history of prior radiotherapy and/or chemotherapy. All patients were staged by the TNM classification according to American Joint Committee on Cancer (AJCC).

The approval was taken from the institutional research committee (GU117) and informed consents were obtained from from all individual participants included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Samples

Surgical resection was performed on all patients under general anestesia. After the surgical resection, tumor and adjacent tumor-free samples from oral epithelial tissue (which are confirmed histologically) with 2-3 mm diameter were put immediately in eppendorf tubes (with 1-2 cc of 0.9% saline) and transfered in liquid nitrogen to the biochemistry laboratory, where the samples were preserved for 6 months at -80 °C temperature until the time of analysis.

Biochemical Measurements

On the analysis day, the tissues were first washed with deionized water to separate blood then homogenized in a homogenizator (Heidolph DIAX 900 model; Heidolph Instruments GmbH & Co. KG, Scwabach, Germany). The upper clear layer was taken to be used in the assays after centrifugation at 5000 g for about 20 minutes.

Measurements of ADA Activity

ADA activity was measured spectrophotometrically by Guisti method [8] which is based on the direct measurement of the ammonia and the results were expressed as unit per mililiter (IU/ml).

Measurement of NOS and NO Activity

NOS activity and NO pool ($NO.+NO_2-$) were measured as described, respectively. NOS activity method is based on the diazotization of sulfanilic acid by nitric oxide at acid pH and subsequent coupling to N-(1-napthyl-ethylene diamine), which is the modification of a previous study [9]. Measurement of the NO pool (mainly consisting of $NO.+NO_2-$) is also based on the same chemical reaction, in which to a greater extent of nitric oxide (NO.), and to a lesser extent of nitrite anion (NO_2-), give a diazotization reaction with sulfanilic acid. The absorbance of complexone formed with N-(1-napthyl-ethylene diamine) reflects the sum of NO. and NO_2- levels in the reaction medium, which is termed the NO pool in the present study. In this method, sodium nitroprusside is used as the chemical standard and the reaction scheme given for the NOS activity measurement,

except for the incubation of the sample with arginine, is followed [10]. The results were expressed as unit per mililiter (IU/ml) for NOS and milimol (mM) for NO.

Measurement of XO Activity

XO activity was determined spectrophotometically by measuring uric acid formation from xanthine at 293 nm [11]. The results were expressed as unit per mililiter (IU/ml).

Statistical Analysis

The correlation between clinicopathological features and the enzyme, molecular activity levels were studied. Statistical analysis was performed with SPSS for Windows, Version 15.0. Chicago, SPSS Inc. The continuous variables was evaluated by visual (Histogram) and statistical methods (Shapiro–Wilk and Kolmogorov and Smirnov Tests) and it was seen that the data did not follow normal distribution. Thus non parametric tests were used. The results were evaluated statistically by using Wilcoxon Signed Rank Test, Kruskal-Wallis Variance Analysis and Spearman Correlation Analysis Test with statistical significance being accepted at 0.05.

Results

The distribution of localization of the oral cavity squamous cell carcinoma of the patients is given in Table 1. Smoking habit was determined in 70.6% (n 12) of the patients and the habit of alcohol usage was 5.9% (n=1).

Clinical and pathological TNM staging were done pre- and postoperatively. The agreement between clinical and pathological staging was assessed as significant (Cohen's kappa/ κ =1,00/ p<0.001). The numbers and percentages of clinical and pathological TNM staging of the patients are given in Table 1.

The ADA activities were significantly increased in cancer tissues compared with the adjacent tumor-free control tissues of squamous cell carcinoma (p<0.001). NO and NOS activities were decreased but not statistically significant (p=0.070 and p=0.796, respectively). No statistically significant difference was observed in terms of XO activities (p=0.233) (Table 2).

No significant correlations were determined between the activity levels of enzyme-molecule (normal and tumor tissue) and age with Spearman correlation analysis test (Table 3). Clinicopathological T stage was classified as T1, T2 and T3+4 and there was no correlation between the activity levels of enzyme-molecule (tumor tissue) and clinicopathological T stage with Kruskal-Wallis variance analysis test (Table 4).

Table 1. Localization and the TNM staging of the oral cavity squamous cell carcinoma of the patients

Variable	Patients (n=17)	%
Tumor Localization		
Tongue	9	52.9
Retromolar trigon	2	11.8
Lip	3	17.7
Gingiva	1	5.9
The floor of mouth	2	13.3
TNM Staging		
Tumor		
1	4	23.5
2	8	47.1
3	2	11.8
4	3	17.6
Nodal		
0	9	52.9
1	4	23.5
2	3	17.6
3	1	5.9
Distant Metastasis		
0	16	94.1
1	1	5.9

Table 2. The activities of ADA, NO, NOS, XO between normal and tumor tissues

	Normal tissue (IU/ml)	Tumor tissue (IU/ml)	*p*-value*
ADA	6.85 (2.45-10.89)	17.98 (9.78-36.45)	p=0.001
XO	0.16 (0.02-0.23)	0.06 (0.04-0.07)	p=0.233
NO	89 (49-163.8)	69.6 (23-170)	p=0.070
NOS	6.02 (5.20-9.70)	5.63 (4.64-7.78)	p=0.796

Data are shown as median (min-max) values. ADA=adenosine deaminase, NO=nitric oxide, NOS=nitric oxide synthase, XO=xanthine oxidase, *=statistical analysis with the Wilcoxon signed rank test

Table 3. The correlation between the age and the activities of ADA, NO, NOS, XO of the normal and tumor tissues

	Normal tissue *p* value*	Tumor tissue *p* value*
ADA	0.670	0.842
XO	0.913	0.105
NO	0.083	0.132
NOS	0.713	0.459

ADA=adenosine deaminase, NO=nitric oxide, NOS=nitric oxide synthase, XO=xanthine oxidase, *=statistical analysis with the Spearman correlation analysis test

Table 4. The correlation between T stage and the activities of ADA, NO, NOS, XO of the tumor tissues

	ADA	NO	NOS	XO
Asym. Sig. (*p* value)*	0.397	0.805	0.797	0.883

T stage describes tumor staging of 1-4, ADA=adenosine deaminase, NO=nitric oxide, NOS=nitric oxide synthase, XO=xanthine oxidase, *=statistical analysis with the Kruskal-Wallis variance analysis test

Discussion

The biological and molecular mechanisms in head and neck cancer were investigated in many studies in recent years. The results are controversial, may be according to the complexity and alteration of the mechanisms. Risk and predisposing factors such as tobacco, alcohol, poor dentition etc. are common (90-95%) in oral cavity carcinoma cases, especially squamous cell carcinoma [1-4]. Although chronic inflamation, free oxygen radical formation, activation of oncogenes and DNA damage are the main steps, pathways are variable for different types of cancer.

In this context, enzyme studies performed with the tumoral and peritumoral tissues are valuable and thought to be effective to understand the carcinogenesis process especially relation with oxidative stress. Although recent studies were limited to blood, serum, secretions etc. and especially focused on as the diagnostic, prognostic or follow-up parameters. In the present study, the role of oxidative stress is elucidated in relation to ADA, NO, NOS and XO activities in patients with oral cavity carcinoma. To our knowledge, this is the first study concerning the measurement of ADA, NO, NOS and XO activities in tumor tissues of patients with oral cavity carcinoma.

ADA is the key enzyme in purine salvage pathway of mammalian, that catalyzes the conversion of adenosine to inosine and deoxyadenosine to deoxyinosine. Due to the irreversibility of the reaction, it is the one of rate-limiting steps in adenosine degradation [6, 7]. Adenosine is present in the interstitial fluids at low levels in physiological conditions. In pathophysiological conditions such as ischemia, inflammation, trauma etc., it can rapidly increase as a result of releasing from intracellular space. It behaves as an 'alarm' that constitutes various responses to restore tissue homeostasis. In the acute phase, adenosine activates pathways that aim to promote healing process however in the chronic phase, it may trigger immune supression or promote unremitting wound healing process such as fibrotic remodelling. In the carcinogenesis process, the increase of adenosine is not a passive product of cancer tissues [12]. It not only generates 'an immunosupressed niche' to promote the onset of neoplasia, an angiogenic and matrix remodelling environment but also activates tumor progression and metastasis indirectly [13, 14].

ADA enzyme activities in head and neck cancer tissues have presented controversial results in literature review [15-19]. Altered purine metabolism in various cancer types which is affected actively by genetic alterations may be one of the reasons for this discrepancy [20]. Ashok *et al.* [15] reported that serum ADA levels in head and neck cancer cases was significantly increased and a highly significant correlation was found between the serum ADA activity and the stage of the disease. After the treatment of the disease by different modalities, the

serum ADA levels were determined to be decreased [15]. Few published clinical studies about the activity of ADA in the oral cavity carcinoma are available in the literature. In the study by Rai *et al.* [16] salivary ADA activity in patients with squamous cell carcinoma of tongue was assessed significantly increased compared with the control group and as the disease stage progressed from stage I to stage III in both genders. In another study by Kelgandre *et al.* [17] statistically significant increase in serum ADA levels was observed in oral cavity carcinoma cases compared with the control group. Also serum ADA levels increased significantly with the histopathological grade. They suggested that serum ADA levels in squamous cell carcinoma of tongue and oral cavity carcinoma might be a useful diagnostic and prognostic biomarkers in clinical practice [16, 17]. In contrast Saracoglu *et al.* [19] found that ADA was significantly lower in saliva of the patients with oral cavity cancer compared with larynx cancer patients and control subjects. However, a statistically significant difference in preoperative and postoperative activity levels of ADA were not observed in patient groups. The low ADA activities in patients with the oral cavity cancer were reported as a compensatory mechanism to the high metabolism of purine and DNA [19, 20]. Our study reveals that ADA activity was increased in the tumor tissues of the patients with oral cavity carcinoma. Our results are in agreement with the studies that showed high ADA activity in patients with various head and neck cancer types [15-17] as a compensatory mechanism against toxic accumulation of its substrates. However there was a significant correlation between serum and salivary ADA activity and tumor stage or histopathological grade in the studies [16-18], in the present study no significant correlations were determined between the activity levels of enzyme-molecule (normal and tumor tissue) and age or clinicopathological T stage (Tables 3 and 4). This finding in agreement with some studies [19-20] can be explained as increased ADA activity is independent from the stage of the tumor.

Another substantial finding of our study is no statistically significant difference was observed in terms of XO activities, in spite of increased activity levels of ADA in the same pathway. ADA and XO enzymes are consecutive enzymes that participate in purine salvage pathway. As thought to be a compensatory mechanism against toxic accumulation of its substrates, XO activities is also be expected to increase. Our results can be explained as elevated ADA enzyme activity is not only a compensatory mechanism against toxic accumulation of its substrates but also an active process.

NO has many critical roles as vasodilatation, regulation of wound healing, non-specific immune response to infection, host defense, cytotoxicity, etc. [21]. In carcinogenesis process, the effect of NO depends on the local concentration of the molecule. It can show both tumoral and antitumoral effects like a 'double-edged blade'. In high concentrations NO inhibits tumor growth however in low concentrations contributes to the tumor development [22]. It is known that endogen NO is cytotoxic and induce the death of tumoral cells [23]. NO and NOS assume potential roles in carcinogenesis with regard to their oxidant and antioxidant effects on the process of inflammation. NO is also one of the nonspecific protective factors against pathogenic microorganisms in the oral cavity. However, the excessive amount of NO can cause the destruction of tumor tissue in tumor metabolism. Therefore NO and NOS may be effective in the development of the oral cavity cancers. In the study by Avci *et al.* [24] NO level and NOS activity were found decreased in the malign lesions compared with the benign lesions. They suggested that decrased NO might be an attempt to supress angiogenesis and/or malign lesions might supress NO production for rapid proliferation [24].

In our study NO and NOS activities were decreased in tumor tissue but not statistically significant. This finding is compatible with the knowledge that NOS expression is mainly in peritumoral and tumoral tissues [22], owing to the control samples were adjacent tumor free tissues. The activity in the tumoral tissue may thought to be the result of carcinogenesis and in the peritumoral tissue, inflammation may be the reason.

The Limitation of the Study

However there are some limitations of our study as 3 of the cases that are lip cancer. The etiological factors involved in lip cancer development are different from other oral cavity cancers such as environmental ultraviolet light exposure is the most common factor. Although smoking has also been associated with the development of lip cancer [1], this difference in the etiology cannot be ignored but oxidative stress has been thought to be the common pathway.

Conclusions

ADA enzyme activity of the tumor tissues was found to be increased compared with tumor free tissues in oral cavity carcinoma patients. Adenosine which is a tumor promoting substrate in carcinogenesis process, generates this effect in the onset of neoplasia, progression and metastasis processes [13, 14]. Degradation of this substrate is the main outcome of increased ADA enzyme activity and contributes to free oxygen radicals development.

In this context, elevated ADA activity might be an attempt to supress formation of the immunosupressed niche which promotes the onset of neoplasia and/or to inhibit tumor progression and metastasis. We think that further studies especially animal and invivo studies are needed to clarify the effectiveness of ADA in this field.

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Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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