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Original Research

ROS related enzyme levels and its association to molecular signaling pathway in the development of head and neck cancer

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Abstract: Given the prevalence and annual incidence of cancer, head and neck cancer is affecting more than 600,000 people each year. In this research, it was decided to investigate that which genes are involved and how MPO, NQO1, SOD2 enzyme levels effective to develop of head and neck cancer and for the first time at the tissue level. 35 tumor tissues in all head and neck anatomy and their surrounding tissue (70 in total) were enclosed the research that received surgery. Determination of the apoptosis genes expression levels (Mtch1, Akt1, Caspase3, Caspase9, Bcl2, Mdm2, mTOR) were determined by RT-PCR techniques and the same patients' sample used for ROS associated oxidant-antioxidant system by using MPO, NQO1, SOD2 enzyme levels using ELISA method. According to statistical results, caspase 9 gene was found statistically high expressed in early stage in contrast to late stage (p=0,013). Level of SOD2, NQO1 and MPO was determined and only MPO level was found significantly important on tumor tissues p=0,008). Specially, our findings for high expression of Cas9 on early stage were thought to be the target for treatment with its well-known initiator role of the apoptosis. Our results suggest that the higher level of MPO in tumor tissues and indicates that it has some role on pathology of head and neck cancers. We believe that, our research will lead the proposal in-vivo studies and will open new areas on therapeutic targets.

Key words: Head and neck cancer; Mtch1; Akt1; Caspase3; Caspase9; Bcl2; Mdm2; mTOR; SOD2; NQO1; MPO.

Introduction

Head and neck region is the area that includes anatomical and physiological respiration, important for life quality. Anatomically it's physiological members are larynx, paranasal sinuses, maxillary, nasal cavity, thyroid gland, salivary glands, ear scoop, laryngeal mucosa, retromolar trigon, gingiva, mandibula, hard and soft palate, tonsils, tongue and mouth base, oropharynx, nasopharynx, hypopharynx and the external tract, other skin, muscular and lymphoid tissue (1-3).

Cancer, in general terms, is a disease that begins with the loss of control of cell division processes and ends with deterioration of the balance between unwanted cell loss and apoptosis. Given the prevalence and annual incidence of cancer, head and neck cancer is affecting more than 600,000 people each year, which impact on primarily even the quality of life including respiration process (4-11). Incidence of the head neck cancer is increased in adults ages moreover, more than one tumor as multiple tumors could be developed individual in a person. Not only it is also possible to differentiate tumor forms invasive to preinvasive but also normal tissue might transform to invasive carcinoma (12,13). Head and neck cancer tumors generally occur in the oral cavity, oropharynx, hypopharynx, larynx and histologically 90% of them classified squamous cell carcinoma (5-8,10). However in most head and neck cancers which are diagnosed in late phases, the choice of the treatment are generally multiple and/or combination therapies including surgery, radiotherapy and chemotherapy (5,6).

The researchers are and will be focusing on the new diagnosis for early prediction is because the World Health Organization (WHO) is considering cancer among diseases that can be prevented (14). Regardless of the cause, the diagnosis and treatment of head and neck cancer has become one of the important health issues to solve.

Reactive oxygen species (ROS) could cause to oxidative stress in the metabolism by curruption of biological structure in the cell and lead to activate inflammation process that responsible for most of the disease including cancer (15-20). In oxidative damage mechanism, free radicals with hypoxia activate the inflammation processes and are involved in the development of cancer by triggering the formation damage by disrupting the cellular micro-macro environment, deforming the normal cell respiration and metabolism of xenobiotics (21,22).

The body system takes control of homeostasis by using antioxidant enzyme systems for protection of the ROS products. As the body attempts to maintain equilibrium, any system will also protect itself from oxidative damage of ROS by trying to use antioxidant enzyme systems (23). However, when the protective capacity of the antioxidant enzyme system is broken, mutage-

nesis and DNA damage are started to trigger carcinogenesis and cell death (15-18, 20, 24-27). Some of these antioxidant enzyme systems are superoxide dismutase (SOD), myeloperoxidase (MPO) and nicotinamide adenine dinucleotide (phosphate) Quinone Dehydrogenase 1(NQO1).

It is well known like other cancer type; oxidants and antioxidant enzyme systems have been researched on head and neck cancer (28-30). According to literature especially SOD2 activity has been found statistically high level on cancer patient compared to controls. In addition, when the cancer stage becomes advanced stage catalase and SOD2 activities start to decrease (28-30). It might be hypothesized that protective system could lose the capacity of protection.

Cancer researchers are focusing on signaling pathways, signal-related gene expressions as a consequence of the advancing technology. Targeting signaling pathways and signal-related gene expressions has become popular for enlightenment to disease mechanisms for new therapies for cancers including head and neck cancer. A variety of studies have been studied to find related signaling pathway for head and neck cancer not widely but targeted anatomically a specific regions especially using cell culture techniques (31-33).

It is well known that if ROS associated system is broken or been corrupted in any case, the protective capacity of the system might not be fixed the damage. In this case, DNA damage and DNA damage related system could cause to trigger cell death and carcinogenesis. When MPO, NQO1, SOD2 enzymes are high level in tissues, it means that there is an oxidation process in the system. Oxidation process might also end up with apoptotic way to eliminate the damaged cells. It was a question that what if these enzyme/enzymes are related with the apoptotic process and in which pathway be affected to end this corruption for head and neck cancer

According to knowledge, there could not find any research in the literature that related head and neck cancer (including several tissue regions) and apoptotic signaling pathways and also their comparison with ROS associated oxidant-antioxidant system by using MPO, NQO1, SOD2 enzyme levels. . In this respect, it was to investigate that both ROS related enzyme levels and molecular signaling pathway relation together for development of head and neck cancer for the first time at the tissue level.

Materials and Methods

Patients Selection

35 tumor tissue in all head and neck anatomy (larynx, oral cavity and thyroid) and their surrounding tissues (70 in total) were enrolled the study which received surgery in Istanbul University Faculty of Medicine ENT Clinic. The tissues were collected after obtaining written informed consent from the participants and approval from Istanbul University's Ethics Committee based on World Medical Association Declaration of Helsinki. Patient's medical records, and pathological reports were received to confirm the diagnosis and cancer status. The patients and control group's characteristics of patients and their clinical and demographic data were given on Table 1.

Table 1: Demographic and clinical data of the patients.

Age (years)		58,23±11,55
Sex (Female/Male)		10/25
Tumor Stage		
	T2	37%
	T3	48,6%
	T4	14,3%
Lymph nodes metastasis		
	N0	54,3%
	N1	17,1%
	N2	28,6%
Tumor localization		
	Larynx	57,2%
	Tongue	11,4%
	Maxilla	5,8%
	Oral cavity	5,8%
	Others	19,8%
Histological Type		
	Squamous	80%
	Others	20%
Smoking habits		77,1%
COPD presence		22,8%
WBC (K/uL)		9,11±3,44
The blood urea nitrogen (mg/dl)		23,02±9,94
AST (mg/dl)		20,57±10,54
ALT (mg/dl)		21,48±11,14
TSH (µU/ml)		1,56±1,03

COPD= Chronic obstructive pulmonary disease, WBC= White blood cell, TSH= Thyroid-stimulating hormone; AST= Aspartate transaminase, ALT= Alanine transaminase.

Expression levels of apoptosis markers associated with cancers to determine of the type of cell death

Determination of the molecular type of death; apoptosis markers associated with cancers which is known to play roles in apoptosis (Mtch1, Akt1, Caspase3, Caspase9, Bcl2, Mdm2, mTOR) expression levels were determined by RT-PCR techniques.

Tumor tissue in all head and neck anatomy were homogenized. After homogenization, RNA were isolated by using Total RNA Purification kit (Jena Biosciense, Jena, Germany) due to the instruction. Quality and quantity of RNA was measured by using Nanodrop (Thermo Scientific, USA). The concentration maintained at the optical density (OD) of 260 nm and the purification level was detected at OD ratio of 260 nm/280 nm.

After isolation of RNA, cDNA was synthesis was performed by using suitable oligo (dT) primers (Table 2). Samples were incubated at 65ºC for 5 min. After incubation, reaction mix was prepared with reaction buffer (5X), 10mM dNTP mix, 200u/µl M-MuLV revers transcriptase and 20u/µl RiboLock RNaz.

Levels of SOD2, MPO and NQO1 in head and neck tissues by ELISA

Tumor tissues and their surrounding tissues were homogenized. Levels were determined by ELISA Kit for Myeloperoxidase (MPO), ELISA Kit for Superoxide Dismutase 2, Mitochondrial (SOD2) (YH Biosearch

Table 2. Forward and revers primers.

Genes	Forward Primer (5'-'3)	Revers Primer (3'-'5)
Mtch1	ATTCAGGATCAAGAACCTA	GGTTATAAACAGAAAT
Akt1	GGGCACATTAGATCAA	ATCATCTCGTAATGACCA
Caspase-3	TAGTTGCAATTGAATTAAATTAGGA	TAGAATACACAGTCTTAAGTG
Caspase-9	ATTGTGAACATCTTCAATGG	AGTAGGACACAAAGATGTCA
Bcl-2	TTTAATTGTATTTAGTTATGGCCT	CAATAAACAATTCTGTTGACG
Mdm2	GAATTATTCAGAAATTATGCATCA	CCAAAGAAACTAACACTTCTC
mTOR	GTCTGAACTGAATGAAGATCAA	TCTTTGTAGTGTAGTGCTTT
Cyclophilin A (Housekeeping gene)	CACCATTGCTGACTGTGGAC	GCCTAGCTGGATTGCAGAGT

Laboratory, Shanghai, China) due to the kits instruction.

Statistical analysis

The statistical analyses were performed using the SPSS 21.0 statistical software package (SPSS, Chicago, IL). P values lower than 0.05 were assumed to be statistically significant. Analysis of relative expression data was performed according to the threshold cycle (CT) method. Differences in the fold changes of the tissues samples for SOD2, MPO and NQO1 expressions were analyzed using the Mann–Whitney U test.

Results

35 tumor tissue that received surgery in all head and neck anatomy (larynx, oral cavity and thyroid) and their surrounding tissues (70 in total) were included the study.

There were no statistical difference between Cas3, Mtch1, Cas9, Mdm2, Akt1, Bcl-2 and mTOR gene expression level in tumor tissues and their surrounding tissues (Table 3).

Besides, level of SOD2, NQO1 and MPO were given in Table 4. According to statistical results the only MPO levels were found significantly high level in contrast to surrounding tissues (p=0,008, 95% Confidence interval = 3,74-23,95).

The fold-change of Cas3, Mtch1, Cas9, Mdm2, Akt1, Bcl-2 and mTOR gene expression levels due to early stage and advanced stage on characteristic of tumor tissues in patients were shown in Figure1. Cas9 gene expressions were statistically found high level in early stage compared with late stage (p=0,013, 95% Confidence interval=0,54-4,22). Even though there were not found any statistical results, there were found high

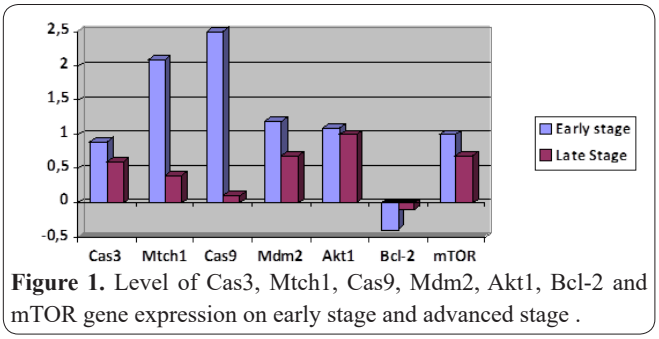

Figure 1. Level of Cas3, Mtch1, Cas9, Mdm2, Akt1, Bcl-2 and mTOR gene expression on early stage and advanced stage .

expression level on Cas3, Mtch1, Cas9, Mdm2, Akt1 and mTOR in early stage except Bcl-2. The only Bcl-2 expression level was found decreasing level.

In addition, lymph nodes metastasis of tumor tissues was compared with the expression of Cas3, Mtch1, Cas9, Mdm2, Akt1, Bcl-2 and mTOR genes (Figure 2). There were found no statistical difference.

Smoking habit as an important factor for head and neck cancer and genes expression were shown on Figure 3. Although there could not have found any important result between smokers and non-smokers, the only

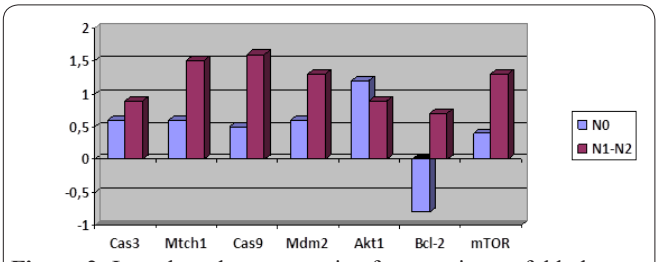

Figure 2. Lymph nodes metastasis of tumor tissues fold changes on expression of Cas3, Mtch1, Cas9, Mdm2, Akt1, Bcl-2 and mTOR genes.

Table 3. Gene expression levels on tumor tissues and surrounding tissues.

	$2^{-\Delta\Delta CT}$ (min-maX)	Fold change	*p*-value	95% Confidence interval
Cas3	5,22(0,09-47,53)	0,78	p>0,05	(-0,21-1,48)
Mtch1	20,71 (0,01-47,26)	1,07	p>0,05	(-1,09-2,48)
Cas9	11,80 (0,03-22,26)	1,02	p>0,05	(-0,22-2,49)
Mdm2	5,13 (0,01-48,33)	0,93	p>0,05	(-0,55-1,66)
Akt1	73,26 (0,01-222,9)	1,10	p>0,05	(-0,57-2,40)
Bcl-2	8,24 (0,01-13,6)	-0,16	p>0,05	(-2,32-0,62)
mTOR	23,88 (0,01-49,8)	0,87	p>0,05	(-0,38-2,26)

Table 4. Level of SOD2, NQO1 and MPO in all tissues (tumor and surrounding).

	Tumor tissue (n=35)	Surrounding Tissue (n=35)	*p*-value
SOD2	12,59±7,36	15,35±5,14	0,07
NQO1	26,33±12,62	30,87±10,12	0,10
MPO	98,46±24,28	84,60±17,43	0,008*

Table 5. Relation of SOD2, NQO1 and MPO levels between tumor stage, lymph nodes metastasis, and smoking habits in tumor tissues.

Tumor Stage		**SOD2 level**	**NQO1 level**	**MPO level**
	Early (n=13)	12,39±7,11	24,96±13,65	95,87±16,21
	Advanced (n=22)	12,71±7,66	27,15±12,24	99,98±28,25
Lymph nodes metastasis		**SOD2 level**	**NQO1 level**	**MPO level**
	N0 (n=19)	13,52±8,12	27,75±13,71	95,18±17,82
	N1-N2 (n=16)	11,49±6,41	24,65±11,41	102,35±30,42
Smoking Habits		**SOD2 level**	**NQO1 level**	**MPO level**
	Smokers (n=27)	11,66±7,22	24,76±11,25	101,21±26,74
	Non-smokers (n=8)	15,73±7,39	31,64±16,20	89,17±8,96

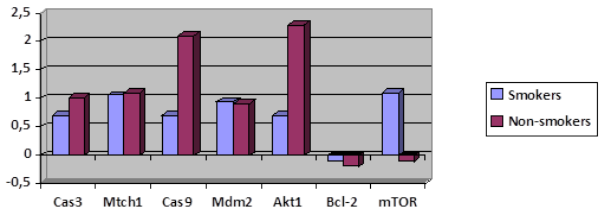

Figure 3. Fold change of Cas3, Mtch1, Cas9, Mdm2, Akt1, Bcl-2 and mTOR gene expressions for smokers and non-smokers.

mTOR, Mdm2 and Bcl-2 levels were found high level of expression on smokers (p>0,05).

Relation of SOD2, NQO1 and MPO levels between tumor stage, lymph nodes metastasis, smoking habits in tumor tissues were given in Table 5. MPO level was observed increased level in smokers (p=0,055).

Discussion

Head and neck cancer including all types is highly effective for daily life comfort such as respiratory, food intake and its treatment options generally are multiple such as combination of surgery, radiotherapy, chemotherapy (5,6). Generally, patients get surgery first to remove the tumor tissue and after surgery if it is necessary radiotherapy and chemotherapy cures could be applied. Even though there are several options for the treatment, the region of head and neck is characteristically capable of recurrence after surgery. Head and neck region have the ability to differentiate their tumor forms even normal tissue might be end up invasive carcinoma (12,13).

The underlying mechanisms of head and neck cancer diseases have not been fully elucidated, but molecular pathway and medical studies are still popular and underway. In our study, the relationship between the target apoptosis pathways and the enzymes for oxidant and antioxidant capacity were investigated with the possibility of a new perspective and creating an infrastructure for the treatment.

The balance of a healthy organism depends on mainly apoptosis. Apoptosis and cell proliferation work inversely proportional such as when apoptosis is decreased cell proliferation starts to increase. The explanation of the decreased number of cells and increased apoptosis means that unwanted tissues and cells are removed in a control manner (34). In this arrangement for the cell apoptotic caspase have the main and active role. Apoptotic caspase are divided into two groups according to their role: initiator caspase and effector caspase. The initiator caspase are caspase 2, caspase 8, caspase 9 and 10 (35,36). These caspase are important markers to understand for starting to cell death. By this role, caspase activation demonstrates that cells work in control manner to eliminate the damaged cell that need to remove for homoeostasis.

In our caspase 9 results have ended up that statistically increased level on early stage compared to advanced stage. Our results suggesting that nevertheless apoptosis is active and effective at the beginning of the disease, it turns out to escape from apoptosis in the advanced stage. We thought that developing activity enhancing drugs or treatments options by targeting the caspase 9 in early stage might be helpful to increase apoptosis before the cancer level up the advanced stage.

Caspase 9 works for eliminating the damaged cell in early stage and have the role during the life period for the disease related proliferation (37,38). Caspase 9 is one of the focuses of cancer studies because its role for the initiate the apoptosis by responsible several reactions such as successful cell death and elimination of damaged/dead cell. Kuida et al have worked with mice that doesn't have caspase 9 activity, they resulted that genotoxic stress level were increased. In addition they have found that the mice without caspase 9 activity has developed resistance to anticancer agents and gamma radiation (39). It is thought that the situation that no caspase 9 activity helps to cancer development and tumor progression. Inadequate apoptosis and caspase 9 activation have the key role for drug resistance on some of cancer type such as ovarian, melanoma, leukemia (40-42). Furthermore, there are some polymorphism studies on caspase 9 and lung, renal, colon carcinoma, the studies have suggested that polymorphisms are related the caspase 9 activation or expression on some level (43-46).

The other aim for our study was to find the correlation of oxidative stress and head and neck cancer. For this purpose, we have worked with MPO, SOD2 and NQO1 level on tissue base. Oxidative stress is the other important research area for cancer. It is well known that any corruption on the protective system from ROS related oxidative stress; it might end up permanent damage. The protective system includes oxidant-antioxidant enzyme system. The corruption on this permanent damage might also responsible for many pathological conditions such as leading cell damage when the sensitive balance is not maintained (18,20). MPO is known with its activation when inflammation occurs and has a role for oxidant formulation (47). There are several cancer studies such as breast, gastric and gynecological cancer have

pointed out that MPO level were found high level (48-50). In gastric carcinoma study, researchers have found the MPO concentration was higher level in patients group than control group (48). The researchers have also worked with the gynecological cancer patients and came up with the similar results that MPO concentration was found statistically higher level compare to controls (49). There is another study that selectin and MPO were studied in the same time in breast cancer patients. The researchers suggested that MPO concentration was again increased in patients before and in course of chemotherapy. In addition, oppositely, after 12th week with chemotherapy treatment, MPO level was started to be decreased it might be because of chemotherapy effect (50). Similarly with the literature, in our study, MPO level was found statistically high level in patients compared with controls.

It is thought that the MPO activation might be related increased the cancerous process leading the cells are dominated by an oxidant activity instead of continuing the apoptosis. This might be the reason of caspase 9 level decreased in advanced stage while increased in early stage in our head and neck cancer patients. It is suggested that increasing the activity of caspase 9 in the early stage might be contributed positively the new perspective for treatment process with detailed molecular analyzes, enlighten molecular pathway and large-scale patients group.

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Interest conflict
The authors declare that they have no conflict of interest.

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