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Review Article

OXIDATIVE STRESS BASED-BIOMARKERS IN ORAL CARCINOGENESIS: A REVIEW

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ABSTRACT

Human cancer development is a multistep process. A variety of endogenous and exogenous stimuli trigger a complex series of cellular and molecular changes that contribute to cancer formation, one of which is the creation of reactive oxygen species (ROS). Oral cancer is one of the most deadly health issues that humanity faces today. Oral cancer accounts for 2% to 3% of all cancers and is the 5th most frequent disease worldwide, according to the World Health Organization (WHO). Oxygen derived species such as hydrogen peroxide, superoxide anion radical, hydroxyl radical ($OH^{\bullet}$), and singlet oxygen are well known to be cytotoxic and have been implicated in the etiology of a wide array of human diseases, including oral cancer. Various carcinogens may also partly exert their effect by generating reactive oxygen species (ROS) during their metabolism. Mutations can result from oxidative damage to cellular DNA, which could have a role in the onset and advancement of multistage carcinogenesis (including mouth cancer) via a variety of processes. ROS has an impact on central cellular processes such proliferation, apoptosis, and senescence, all of which have been linked to cancer development. Antioxidant deficiency or an oxidant-antioxidant imbalance can cause oxidative damage to cellular macromolecules, which can lead to cancer. Understanding the significance of reactive oxygen species (ROS) as essential mediators in signaling pathways may open up new avenues for pharmaceutical intervention. We review the current state of knowledge on the role of these oxidative modified cellular byproducts in serving as reliable biomarkers for oral cancer detection, prognosis and diagnosis.

Keywords: Reactive oxygen species, Oral cancer, Oxidants, Antioxidants, Free radicals, Cancer biomarkers, Oral carcinogenesis.

1. INTRODUCTION

1.1. Oral cancer

Cancer is one of the most common causes of morbidity and mortality today, with more than 10 million new cases and more than 6 million deaths each year worldwide. It is estimated that around 43% of cancer deaths are due to alcohol consumption, tobacco use, diet, lifestyle habits and infection. More specifically, tobacco consumption can cause cancers of the oral cavity, pharynx, larynx, oesophagus, etc. In addition, tobacco consumption and exposure to environmental tobacco smoke (passive smoking) both have shown to increase the risk for lung cancer incidence. Finally, tobacco use and alcohol consumption act synergistically to cause cancers of the oral cavity, pharynx, larynx and oesophagus [1]. Oral cancer refers to a subset of head and neck cancers that arise in the lips, tongue, salivary glands, gingiva, mouth floor, oropharynx, buccal surfaces, and other intra-oral areas. Oral squamous cell carcinomas (OSCC) account for more than 90% of oral cavity malignancies [2]. From a clinically identifiable pre-cancer stage, some patients develop OSCC. These conditions are collectively identified as oral potentially malignant disorders (OPMD). OPMD are defined as clinical presentations that carry an increased risk to develop into OSCC [3]. Leukoplakia, erythroleukoplakia, oral lichen planus, and oral submucous fibrosis are also common OPMD disorders. Lip and oral cavity cancers resulted in more than 177,000 fatalities and more than 350,000 new cases in 2020, according to worldwide health statistics [4]. The prevalence of OPMD has been estimated to be 4.47 percent worldwide [5]. Asia was responsible for more than two-thirds of OSCC [6]. OSCC was the 12th most prevalent cancer type in Asia in 2012; by 2018, it has risen to the 11th position, indicating an upward tendency over time [7]. Compared to other cancers, OSCC demonstrate low five year survival rates, the survival rate is about

20% when diagnosed at advance stage and it can improve up to 80% when diagnosed at early stages [8]. The 5 year survival rate has not improved with time despite advances in treatment [9, 10]. Early detection is important to reduce mortality and morbidity associated with this disease. Lack of effective screening protocols was highlighted as a major barrier for early detection [11]. Identifying which OPMDs will develop into a malignancy remains a challenge, as the malignant transformation of OPMD is not consistent [12]. Hence, the need of biomarkers for screening, diagnosis and prognosis in OSCC and OPMD has been emphasized [13, 14].

1.2. Concept of oxidative stress and ROS

Oxygen is a highly reactive atom that can combine with other elements to form potentially harmful compounds known as free radicals. A free radical is a chemical entity that has one or more unpaired electrons. All highly reactive, oxygen-containing molecules, including free radicals, are referred to as reactive oxygen species (ROS) [15]. Various free radicals are Hydroperoxyl (per hydroxyl) radical, Superoxide radical, Hydrogen peroxide, Singlet oxygen and triplet oxygen, Nitric oxide radical (NO^-), Peroxynitrite ($OONO^-$) and Hypochlorite radical ($HOCl^-$) [16]. All are capable of reacting with membrane lipids, nucleic acids, proteins, enzymes and other small molecules; resulting in cellular damage [17].

1.2.1. Mechanism of action of free radicals

ROS can cause tissue damage by a variety of different mechanisms which includes DNA damage, Lipid peroxidation (through activation of cyclooxygenase (COX) and lipoxygenase pathway), Protein damage including gingival hyaluronic acid and proteoglycans, Oxidation of important enzymes, for example, antiprotease such as, 1 antitrypsin, Stimulate proinflammatory cytokine which are released by monocytes and macrophages by depleting intracellular thiol compounds and activating nuclear factor kappa beta [17].

1.2.2. Oxidative stress

The idea of oxidative stress, developed by Sies [18], can explain the link between free radicals and disease. Oxidative stress is described as a condition in which the body's oxidative systems outnumber its antioxidant systems due to a breakdown of equilibrium between them [19]. Biomarkers of oxidative stress are products of such cellular damage [20]. The oral cavity is sensitive to reactive oxygen caused by inhalation of oxidizing chemicals in tobacco smoke and air pollution, in addition to reactive oxygen generated by the host tissue. The realization that ROS (free radicals) and oxidative stress play important role in the etiology and progression of major human degenerative diseases has triggered enormous and worldwide interest in endogenous and exogenous antioxidants [19].

1.2.3. ROS

Endogenous and external chemicals can both cause ROS. Mitochondrial metabolism, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation are all potential endogenous sources [21]. Environmental agents such as non-genotoxic carcinogens, different xenobiotics, ultrasound, and microwave radiation are examples of exogenous sources [22, 23]. They have a dual nature: on the one hand, they are required for regular biological functioning, yet in excess; they can harm cells and lead to cancer.

1.2.4. Mechanism of action of ROS in cancer

Cancer development is characterized by summative action of multiple events occurring in single cell. It can be described by three stages: Initiation, promotion, and progression. ROS is involved in all these stages. The effect of oxidative stress at a certain stage of carcinogenesis is directly proportionate to the type and the reactivity of radicals involved. Initiation results when a normal cell sustains a DNA mutation that, when preceded by a round of DNA synthesis, results in fixation of the mutation, producing an initiated cell. Initiation of cancer by ROS is supported by presence of oxidative DNA modifications in cancer tissues [24]. The promotion stage is characterized by clonal expansion of initiated cells, by induction of cell proliferation and/or inhibition of apoptosis [22]. Oxidative stress is strongly involved in this stage. ROS can stimulate expansion of mutated cell clones by temporarily modulating the genes which are related to proliferation or cell death [25] and by regulating activity of certain transcription factors such as nuclear factor-jB (NFjB), Nrf2, HIF, and p53; [26] which control cell growth and oncogenesis [27]. It can lead to NFjB activation, with subsequent induction of genes encoding for proteins that inhibit apoptosis [28]. It can also act at signal transduction level to exert prosurvival functions. Oxidative stress can activate ERK/MEK and PI3K/AKT pathways. This could result in inactivation of proapoptotic proteins and

up regulation of antiapoptotic genes [29]. A low level of oxidative stress can stimulate cell division in promotion stage, and thus promotes tumor growth [30]. This implies that ROS production during this stage is the main mechanism of ROS related tumor promotion. ROS also contributes to the last stage of carcinogenesis; progression. In this stage, generation of large amounts of ROS may contribute to mutate, inhibit antiproteisases, up regulate matrix-metalloproteinases (MMPs) [31, 32] and injure local tissues. Increased levels of oxidatively modified DNA bases may contribute to genetic instability and metastatic potential of tumor cells in fully developed cancer [33]. ROS is reported to be crucial for triggering angiogenic response, which is important in cancer metastasis [34]. This suggests that ROS is involved in all these stages of carcinogenesis. ROS, which are formed through various events and pathways, react with and damage cellular components and contribute to neoplastic transformation [35, 36].

1.3. Oxidative DNA damage and cancer

Damage to DNA by ROS has been widely accepted as a major cause of cancer. ROS can damage DNA and the division of cells with unpaired or misrepaired damage leading to mutations. The majority of mutations induced by ROS appear to involve modification of guanine, causing G→T transversions [37], single strand breaks, and instability formed directly or by repair processes. In human tumors, G to T transversions is the most frequent mutations in the p53 suppressor gene [38]. Elevated levels of modified bases in cancerous tissue may be due to the production of large amount of H2O2, which has found to be characteristic of human tumor cells. A study supported initiation of cancer by the presence of oxidative DNA modifications in cancer tissue [39].

1.4. Oxidative stress in oral carcinogenesis

Cigarette smoking, alcohol consumption and betel quid (BQ) chewing have been identified as the most important factors in causing oral cancer through the induction of oxidative stress and more precisely generation of ROS [40-44]. It is well documented that ROS play an important role in the development of cancer [45]. Their accumulation (with simultaneous impairment of the intracellular enzymatic and non-enzymatic antioxidant defense systems) is able to cause a number of oxidative modifications to several macromolecules including proteins, lipids and DNA all of which could ultimately lead to tumor development [46, 47]. There is a strong link between tobacco-cigarette smoke and oral cancer suggesting that smoking is responsible for 50-90% of all oral cancer cases worldwide [48]. In general, cigarette smoke contains more than 4,000 different compounds with more than 60 of them being potentially carcinogenic namely polycyclic aromatic hydrocarbons (PAHs), aromatic amines, benzene, aldehydes, N-nitrosamines, etc. In addition, molecules like alcoxyl, peroxyl and hydroxyl radicals, hydrogen peroxide and the superoxide anion are also generated in tobacco smoke [49]. To this end, smokers are thought to be in a continuous state of oxidative stress generation and thus at a higher risk to develop oral cancer, although the exact mechanism is not yet fully understood [40]. Numerous studies have determined a relationship between tobacco smoke, ROS and oral carcinogenesis reflected by the presence of protein oxidation, lipid peroxidation and oxidative DNA damage byproducts [42, 50, 51]. Alcohol consumption is the second leading risk factor (following tobacco smoke) in the etiology of oral carcinogenesis with the risk increasing with duration and intensity of alcoholic consumption [52]. For example, individuals who consume 4-5 drinks per day, have a 2- to 5-fold higher risk of developing oral cancer when compared to non drinkers [53]. Although tobacco smoking and BQ chewing have been shown to act in a synergistic manner in inducing higher rates of oral cancer [54], such synergism has not been observed between alcohol consumption and tobacco smoking irrespective of drinking duration and frequency [55]. In general, ethanol when ingested is oxidized to its principle metabolic product, acetaldehyde (AA), by the enzyme alcohol dehydrogenase (ADH); whereas the enzyme aldehyde dehydrogenase 2 (ALDH2), catalyses the oxidation of acetaldehyde to acetate [41, 56]. Finally, when alcohol consumption reaches high levels, its oxidation is mediated by cytochrome P450 (CYP) and specifically CYP2E1. The link between alcohol consumption and increased risk of oral cancer has been based on AA's cytotoxicity and adductforming capacity as well as cytochrome P450's function which is involved in the oxidation of ethanol to AA with the concomitant production of ROS that further interact with cellular targets enhancing oxidative damage [41, 57]. Lipid peroxidation (e.g. malondialdehyde; MDA, crotonaldehyde; Cro, acrolein and 4-hydroxynonenal; 4-HNE) and AA-induced protein adducts have been reported in patients with oral cancer and oral

leukoplakia (a premalignant lesion), both of which are characterized by prolonged and excessive alcohol intake [58]. BQ chewing represents a habit for about 600 million people with South-East Asia and the Pacific Islands being the most prevalent areas [53]. BQ represents a variety of compounds such as the leaves of *piper betle L*, lime, catechu (extract of *Acacia catechu*), slaked lime and husks of the *areca catechu* [53, 59]. Although BQ has been classified as a carcinogen, the exact mechanisms underlying its connection to cancer development have not been clarified. However, it is known that BQ chewing is associated with premalignant oral diseases such as leukoplakia and submucous fibrosis, as well as with oral cancer (especially when in conjunction with cigarette smoking) [59, 60]. Moreover, it has been reported that BQ chewing is associated with the generation of ROS, as aqueous extracts of *areca catechu* have been shown to be responsible for the production of the superoxide anion and hydrogen peroxide when in the presence of alkaline pH. In addition, in samples of slaked lime, used by BQ chewers, the presence of calcium hydroxide was strongly associated with the generation of ROS as well as oxidative DNA lesions like formation of 8-hydroxyguanosine induced by the areca nut [61]. Although it is documented that long-term chewing of BQ is associated with oral cancer development, several reports support its non-prolonged and frequent consumption (even in the presence of cigarette smoking) does not contribute to the high incidence of oral carcinogenesis [59].

1.5. Oxidative stress-based biomarkers in oral cancer

A biomarker is defined as a characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention [62]. High rates of morbidity and mortality associated with low levels of prognosis and diagnosis for oral cancer impose the development for useful biomarkers, in order to improve rate of survival, early detection and quality of life. In the field of biomarker discovery specific to oral cancer, body fluids (such as saliva and blood serum) have gained much attention and are hypothesized to serve as important tools at this scope [63]. Productions of ROS are involved in carcinogenicity through mechanisms including protein oxidation, lipid peroxidation and oxidative DNA damage [43, 46] all of which could potentially serve as reliable biomarkers in oral cancer. To this end, such use of oxidative stress biomarkers can be of paramount importance in enhancing detection, diagnosis and prognosis of oral carcinogenesis. In general, ROS are able to react with the DNA backbone, causing oxidative damage such as apurinic and/or apyrimidinic DNA sites, single (SSBs) and/or double strand breaks (DSBs), oxidized purines and/or pyrimidines [64] and non-DSB oxidatively-induced clustered DNA lesions (OCDLs) [65]. More specifically, hydroxyl radicals directly target DNA, causing site-specific damage whereas hydrogen peroxide is involved in the production of oxidized bases through Fenton and Haber-Weiss reactions [66]. Formation of 8- hydroxyguanosine (8-oxo-dG), is the most studied and abundant oxidative DNA lesion (used as a specific biomarker of oxidative DNA damage) that is characterized by inducing G⟶T transversions which are mutagenic [67]. Immunohistochemical analysis in patients diagnosed with OSCC (stage III) revealed that levels of 8-oxo-dG were significantly elevated when compared to control individuals [50]. In the same comparative study, similar results were found in a DMBA (7, 12-dimethylbenz (a) anthracene) model, a substance that induces carcinomas similar to human OSCC-induced hamster buccal pouch. Furthermore, in a recent report including patients with oral leukoplakia, lichen planus and submucous fibrosis, increased levels of 8-oxo-dG were determined which were subsequently alleviated after administration of curcumin [68]. Finally, it is known that 8-oxoguanine DNA glycosylase (OGG1) is the essential enzyme that counterbalances excessive levels of 8-oxo-dG lesions and thus protects against cancer [69]. To this end, salivary analysis of patients with OSCC showed that OGG1 enzymatic levels were significantly reduced indicating a correlation between enhanced levels of 8-oxo-dG and oral cancer development, suggesting OGG1 as a prominent oxidative stress biomarker [70]. Besides DNA, ROS can react with other biological molecules. Proteins can also be targets of ROS during conditions of oxidative stress generation. As proteins regulate cell structure and several signalling pathways and enzymatic processes, their oxidation state is of critical importance to cellular homeostasis [71]. In fact, it is known that protein oxidation is correlated with changes in its conformational status, accompanied by elevated hydrophobicity, denaturation, aggregation and precipitation that ultimately leads to cell death [67]. Additionally, it is believed that certain proteins are more sensitive to oxidation, due to:(i) the presence of oxidation-sensitive amino acids residues, (ii) their

localization in the cell (iii) The presence of metal binding sites [68].

Protein oxidation by ROS is mediated through several routes including the following:

- Cleavage of peptide bonds (through interaction of hydroxyl radicals with proteins) resulting in the formation of alkyl radicals, alkyl-peroxide radicals, alkyl peroxide and alkoxyl radicals,
- Direct modification of several amino acids (especially those with aromatic side chain groups and sulfhydryl residues) like arginine, histidine, lysine and proline,
- Damage of the polypeptide backbone *via* reactions including metals such as Fe and Cu, hydrogen peroxide, and resulting hydroxyl radicals production [71].

Protein carbonylation is the most important product of protein oxidation and it is characterized by the formation of aldehydes and ketones that are able to react with 2, 4-dinitrophenylhydrazine (DNPH), resulting in the formation of hydrazones, during the oxidation of amino acids as threonine, proline, lysine and arginine [71, 72]. An alternative pathway for the formation of protein carbonyls is the reaction of DNPH with lipid peroxidation products, such as MDA, acrolein and 4-HNE [73]. Protein carbonylation is an irreversible condition, probably due to the oxidative stress-induced impairment of proteolytic processes (proteasomes) leading to accumulation of carbonylated proteins and consequently cytotoxicity [47]. In a recent study, protein carbonylation was used as a protein oxidation biomarker in OSCC patients displaying increased levels of carbonyls in saliva samples, compared to healthy individuals [70]. Additionally, another OSCC study demonstrated significantly higher levels of salivary carbonylation, when compared with healthy individuals. It is noteworthy that these patients had no history of smoking or alcohol consumption [74]. Finally, in patients with oral pre-cancerous conditions (e.g. leukoplakia) and OSCC (drinkers with more than 10- years of excessive alcohol consumption, and current smokers) an increase of protein adducts in samples of oral biopsy was observed [58]. Lipids and more precisely phospholipids (present in cellular membranes) are another susceptible target for reactive oxygen species [75]. More specifically, polyunsaturated fatty acid moieties of membrane phospholipids are the major site of ROS action in a reaction that includes the extraction of a hydrogen atom from an allylic or a bis-allylic location of polyunsaturated fatty acids [67]. The resultant oxidation of lipids (lipid peroxidation; LPO), involves the production of reactive aldehydes such as MDA, Cro, acrolein, and 4- HNE [66]. The former two, are the most important aldehyde products of LPO that can further react with proteins as well as the DNA backbone forming even more adducts. Finally, while MDA is associated with a range of mutagenic properties, 4-HNE is known to confer cytotoxicity [76]. A recent study has shown that increased MDA blood levels were associated with increased levels of oxidative stress in patients with OSCC and that such an increase of MDA levels results in the direct decomposition of polyunsaturated fatty acids in membrane phospholipids. Moreover, all these OSCC patients were either tobacco chewers or cigarettes smokers indicating the close relationship between tobacco use and oral cancer development. In another study, MDA levels were shown to be higher in oral cancer patients when compared to healthy individuals [77]. Finally, a series of studies have shown evidence of elevated MDA levels (detected in both salivary and blood samples) in patients diagnosed with oral lichen planus [78], oral leukoplakia and submucous fibrosis [68] indicating the presence of this lipid oxidation biomarker in premalignant lesions as well. On another note, the presence of antioxidant systems (enzymatic and non-enzymatic) acts as a defence mechanism in order to minimise oxidative cellular damage. The enzymatic antioxidant scavenging system of intracellular ROS levels comprises enzymes as superoxide oxidase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione transferase (GST). Non-enzymatic antioxidant systems include those of vitamins C and E, reduced glutathione, etc. These members of the antioxidant systems are currently used as 'indirect' biomarkers of oxidative stress generation as their reduced levels imply an overall state of an intracellular oxidative environment. To this end, a recent study has shown reduced total levels of antioxidant enzymes (e.g. GPx, SOD, vitamins C and E, etc.) in blood samples of patients with tongue carcinoma who were also tobacco users. Furthermore, in another study, reduced levels of CAT and SOD were detected in tissue samples from OSCC patients who were also either tobacco chewers or smokers. Finally, reduced levels of serum and salivary vitamins C and E were observed in patients with premalignant lesions of oral leukoplakia, lichen planus and submucous fibrosis [68].

1.6. Antioxidant defense

Endogenous and exogenous antioxidants can prevent and repair damage caused by ROS. Therefore, they are called free radical scavengers and can improve the immune defense and lower the risk of disease and cancer. Enzymatic antioxidants, which include SOD, GPx, and CAT, act by chelating superoxide and other peroxides. They act as endogenous antioxidant defense systems, which clear ROS activity and accumulation in cells and maintain redox balance. The first line of defense against free radicals is SOD, which catalyzes the dismutation of superoxide anion radical ($O2^{\bullet-}$) into hydrogen peroxide (H_2O_2).The formed oxidant H_2O_2 is then transformed into water and oxygen (O2) by CAT or GPx. The selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase regenerates GSH from GSSG, with NADPH. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH). In addition, nonenzymatic antioxidants (Vitamins E and C, coenzyme Q, carotene, and glutathione) serve as an important biological defense from ROS attack [79].

2. CONCLUSION

Assessment of oxidative stress and augmentation of the antioxidant defense system may be important for the treatment and prevention of carcinogenesis. Cigarette smoking, alcohol consumption and BQ chewing have all been identified as important factors in the pathophysiology of oral malignant and premalignant lesions *via* a wide range of underlying mechanisms including ROS-induced generation of oxidative stress. To this end, a number of cellular targets (e.g. DNA, proteins and lipids) have been identified whereby their oxidative-modified byproducts (8-oxo-dG, carbonyl groups, MDA, etc.) could potentially serve as reliable biomarkers of detection, diagnosis and prognosis in oral carcinogenesis. However, there are only a limited number of studies suggesting the use of such biomarkers in the clinical setting and even those utilize a rather restricted number of these oxidative-modified byproducts. There is clearly a need for the design of more elaborate studies for the characterization, evaluation and development of oxidative stress-based biomarkers with clinical significance in the management of oral malignant and pre-malignant lesions.

Conflict of interest

None declared

3. REFERENCES

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