Analysis of CCND1 protein and circulatory antioxidant enzyme activity association in oral squamous cell carcinoma

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A R T I C L E   I N F O

Abstract
Antioxidants are involved in the process of cellular damage prevention, which is considered as an avenue for cancer development. Free radicals are produced in the body upon exposure to stress, cigarette smoke, alcohol, toxins found in personal care products, pesticides in foods, radiation from the sun, viruses, germs or fungi etc. CCND1/CyclinD1 protein was found to be overexpressed in Oral squamous cell carcinoma. One hundred patients with oral squamous cell carcinoma were recruited along with hundred controls for this study from MNJ institute of Oncology with the approval of Ethics Committee, 5 ml blood samples were collected from each patient and centrifuged to collect serum for various assays. The antioxidant enzymes like catalase, SOD, GPX and GST were estimated using enzymatic assays. Results were expressed as unit of activity for mg of protein. In silico analysis is performed using STRING v 11 Protein interaction tool. The patients with oral cancer had significantly reduced activities of SOD, GST and GPX (1.49 ± 0.49, 3.97 ± 0.86 and 10.7 ± 0.73 respectively) compared to healthy controls (4.37 ± 1.43, 6.10 ± 1.12 and 13.8 ± 1.25 respectively) (p < 0.005). However no significant difference was observed with regard to catalase activity (2.71 ± 6.51 and 4.03 ± 1.48) (p = 0.28). The proteins interaction PPI enrichment p-value was found to be 3.22e-10 predicted significantly more interactions. Our research findings shown that there was a decline in activity of superoxide dismutase, glutathione peroxidase and glutathione s transferase in addition, personal habits like smoking play a major role in the development and progression of oral carcinogenesis and based on In silico analysis results CCND1/Cyclin D1 could be the potential therapeutic target in oral squamous cell carcinoma.

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1. Introduction

Cancer currently found to be the second leading cause of mortality globally. Various mechanisms are associated with malignancies due to consumption of alcohol, which causes free radicals generation, procarcinogen activation enhancement, cellular regeneration modulation and nutritional deficiencies. Chronic alcoholism causes atrophy and lipomatous metamorphosis of the parenchyma of parotid and sub maxillary gland, alterations lead to functional impairment of saliva flow, which increases viscosity (Hsuan et al., 2010), and the mucosal surface is insufficiently rinsed, therefore it is exposed to carcinogens. The alcoholic beverages contains various carcinogenic compound, as polycyclic hydrocarbons asbestos fibers and nitrosamines (Boffetta et al., 2011). The process of ethanol oxidation, first conversion of ethanol to acetaldehyde, which leads to inhibition of protein synthesis (Farhad et al., 2010), as acetaldehyde is highly toxic, mutagenic and carcinogenic interferes with the mechanism of DNA synthesis and repair leads to development of tumor (Tramacere et al., 2012), one of the recent studies has shown cigar ate smoking has high impact on lung cancer (Aldakheel et al., 2021).
Free radicals are capable of attacking the healthy cells of the body causing them to lose their structural function. Generation of free radicals is the main factor which causes aging and resulting degenerative diseases as cancer, cataract, decline in immune system, cardiovascular diseases and brain dysfunction (Udensi et al., 2014). Antioxidants play an important role in deactivation of free radicals and maintains optimal cellular functions and systemic health, protection of cells and organ systems of the body against free radicals in humans could be obtained by highly sophisticated complex antioxidant protective system, which includes antioxidants like ascorbic acid, carotenoids etc. (Maria et al., 2014) and antioxidant enzymes like GST, SOD, GPX, lactoferrin, metal binding proteins like ferritin, (Saharia et al., 2014) and various antioxidant phytonutrients.

Oxidative damage to proteins, DNA, and various macro molecules had been found to be involved in pathogenesis of cancer (Hsueh et al., 2014), whereas, oxidants causes stimulation of cell division, which is considered to be a critical factor in generation of mutagenesis. When a cell divides with damaged DNA strands cell metabolism and duplication becomes unbalanced, which causes mutation, which is an important factor in the development carcinogenesis (Ozben, 2015). Both cigarette smoking and chronic inflammation are two major factors which causes cancer, as strong free radical components are involved in their mechanism of action.

The mechanism by which alcohol consumption exerts its carcinogenic effect have not been defined completely although plausible events include genotoxic effects of acetaldehyde the main metabolite of ethanol. A causal association has been established between alcohol consumption and cancer of oral cavity. Evidences suggest that the effect of alcohol is modulated by polymorphism in genes encoding enzymes for ethanol metabolism, folate metabolism and DNA repair.

Cyclin D1 protein encodes the gene CCND1 located in chromosomal band 11q13, helps in cell cycle proliferation during G1-S phase. Oral squamous cell carcinoma development is found to be linked with over expression of Cyclin D1 (Saawarn et al., 2012; Santarius et al., 2010). A previous study reported that, the polymorphism of 870A allele of CCND1 found to be associated with individual cigarette smoking history (Duan et al., 2013).

2. Materials and methods

2.1. Approval from Ethics Committee

Ethical clearance was obtained from Institutional Ethics Committee of MNJ Institute of Oncology, Regional Cancer Centre-Hyderabad Before the sample collection the study was explained, and participants were asked to complete an informed consent form.

2.2. Patient selection & Inclusion and exclusion criteria

One hundred patients with oral squamous cell carcinoma (OSCC) were included in the study and one hundred controls participants. Participants in this study were selected from MNJ institute of oncology and regional cancer center during the years 2013 and 2014. Children and pregnant women are excluded from the study.

2.3. Sample collection

In this study, 5 ml of blood was collected from each subject by venous arm puncture between 8am – 10am to avoid circadian variations. Smoking volunteers were asked not to smoke for one hour prior to the sample collection. The blood was centrifuged and serum was collected in eppendoffs and stored immediately at −80°C for further analysis.

2.4. Determination of antioxidant enzyme activity in serum samples of patients:

2.4.1. Superoxide dismutase activity

The specific activity of antioxidant enzyme superoxide dismutase (SOD) was estimated spectrophotometrically (Marklund et al., 1974). The total reaction was carried out at 25 °C for 3 min. Change in absorbance at 420 nm with suitable blank was also recorded. One unit of the SOD activity is defined as the amount of the enzyme required to inhibit 50% of auto-oxidation of pyrogallol (Marklund et al., 1974).

2.4.2. Catalase activity

Catalase activity was assayed spectrophotometrically (Sinha, 1972). The reaction mixture was measured at 570 nm against control and the activity was expressed as units per milligram protein (one unit is the amount of enzyme that utilizes 1 mmol of H2O2/ min).

2.4.3. Glutathione peroxidase activity

The principle of this method is that the rate of glutathione oxidation by H2O2, as catalyzed by the GPX present in the supernatant is determined and the color was read against a reagent blank at 412 nm (Lawrence et al., 1974).

2.4.4. Glutathione-s-transferase activity

The reduced glutathione was estimated by the method of (Ellman, 1959). Total reaction was measured at 412 nm against blank and values were compared with a standard curve of GST.

2.5. Bioinformatics analysis

The String v11 (https://string-db.org/) is used for retrieval of protein–protein interaction. CCND1 protein is used in the present study to analyze the proteins interaction with cyclin D1/CCND1.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6. Data has been expressed in terms of mean ± standard deviation. Significance was evaluated via Student’s t-test, with P ≤ 0.05 considered as significant.

3. Results

3.1. Clinical Characteristics OSCC patients included in the study

Between the age group 46–65 years highest percentage of OSCC patients were identified. The OSCC male patient’s age range between 26 and 74 years and female was between 21 and 63 years. In the control group 102 healthy subjects males number 64 and age range 20–70 years, females control group number 38 and age range of 20 – 50 years. The percentage of primary tumor site in predominance of buccal mucosa (BM) was found to be (37.33%), tongue (22.0%), mandible and oral cavity was 12%, and 10% respectively.

In our present study, the staging clinical stage III showed the highest frequency (40%), stage IV (31.33 %),stage II (22%) and the stage I (6.67 %) showed very low frequency in contrast to other groups (Table 1). All tumors were confirmed by histopathological analysis.
3.2. Antioxidant enzymes in cases and controls

The patients with oral cancer had significantly reduced activities of SOD, GST and Gpx (1.49 ± 0.49, 3.97 ± 0.86 and 10.7 ± 0.73 respectively) compared to healthy controls (4.37 ± 1.43, 6.10 ± 1.12 and 13.8 ± 1.25 respectively) (p < 0.005). (Shown in Figure 1, 2, &3). However no significant difference was observed with regard to catalase activity (2.71 ± 6.51 and 4.03 ± 1.48) (p = 0.28) (shown in Figure 4).

3.3. Bio information analysis of CCND1 protein, associated with oral squamous cell carcinoma

**CCND1 sequence retrieval**

The amino acid sequence of CCND1 protein has been retrieved from RCSB protein data bank (https://www.rcsb.org/).

**STRING v 11 analysis of CCND1 protein**

STRING available online at (https://string-db.org/) has been used to interpret the interaction of CCND1 protein with the pro-
teins of homo sapiens as organism involved on the biological activity (Fig. 5).

The number of nodes was found to be 11, each node represents all the proteins produced by a single protein-coding gene locus and number of edges was found to be 49, edges represent protein–protein associations that means proteins jointly contribute to a shared function they are not just binding physically each other and the PPI enrichment p-value is 3.22e-10 found to have significantly more interactions (Fig. 5).

Predicted functional proteins interacted with the CCND1 protein using STRING v II

The ten functional proteins were predicted with homology score from 0.999 to 0.992. CDK4, CDK6, CDK2 and CDKN1A predicted homology score was found to be 0.999, where as CDKN1B was found to be 0.996 and the least score was predicted for STAT3 and IFNAR1 was found to be 0.993 (Table 2).

| S. No | Predicted functional proteins | Homology score |
|-------|--------------------------------|----------------|
| 1.    | Cyclin-dependent kinase 4 (CDK4) | 0.999          |
| 2.    | Cyclin-dependent kinase 6 (CDK6) | 0.999          |
| 3.    | Cyclin-dependent kinase 2 (CDK2) | 0.999          |
| 4.    | Cyclin-dependent kinase inhibitor 1 (CDKN1A) | 0.999 |
| 5.    | Cyclin-dependent kinase inhibitor 1 (CDKN1B) | 0.996 |
| 6.    | Catenin beta-1 (CTNNB1) | 0.995          |
| 7.    | Signal transducer and activator of transcription 3 (STAT3) | 0.993          |
| 8.    | Interferon alpha/beta receptor 1 (IFNAR1) | 0.993          |
| 9.    | Retinoblastoma-associated protein (RBI) | 0.992          |
| 10.   | Cyclin-dependent kinase inhibitor 1C (CDKN1C) | 0.992          |

4. Discussion

A numerous research studies reported that, chronic alcohol consumption found to be the main cause of carcinogenesis, as there is a correlation exist between alcohol ingestion and occurrence of cancers (Ahmedin et al., 2014) and due to alcohol consumption nutritional status would be impaired leads to malnutrition, moreover, alcohol facilitates in uptake carcinogens from tobacco smoke via cell membranes that are damaged and alter in their molecular composition. Tobacco produces deleterious oral health consequences. Its effects are equally serious on the general health of users. The risk of mortality is significantly higher in tobacco smokers and chewers compared to non-users of tobacco (Stefanopetti et al., 2009; Kroll et al., 2012). Our study also shows that the risk of oral cancer is significantly high with respect to smokers and chewers when compared to alcoholics.

The cells damaged due to free radicals play a pivotal role in formation of carcinogenesis, epidemiological evidences reported that, low antioxidant intake or low antioxidants level in blood increases the cancer risk as oxygen is highly reactive which is capable of potentially damaging molecules usually called as free radicals (Jaonna et al., 2014). Fortunately, various beneficial compounds known as antioxidants control free radical formation naturally. When the availability of antioxidants is limited that this damage can become cumulative and debilitating. Antioxidants helps in deactivation of free radicals prior to their destructive effects on cells and antioxidants are useful in maintenance of cellular and systemic health (Ozben, 2015). In this study, we observed an association between low antioxidant levels and carcinogenesis in south Indian subjects.

It was estimated that diet might account for as much as 35% of all human cancers (Eboh et al., 2014). Low dietary intake of fruits and vegetables doubles the risk of most types of cancers (Perni et al., 2014). Antioxidants, antioxidant supporting systems, and nutritional modulators supports mitochondrial function (Surinder et al., 2015). It has been suggested that mitochondrial dysfunction is related to damage caused by ROS produced because of increased oxidative stress. Levels of ROS produces within the mitochondria are reported to increase with age (Luca et al., 2015). In our study, we observe correlation between malnutrition and carcinogenesis. The risk of oral cancer was noticed in middle age group subjects when compared to young and old age subjects.

Cyclin D1 (CCND1) protein plays an important role in cell cycle proliferation helps in transition from G1 to S phase during cell division, in contrast to our Insilico analysis using STRING search tool...
for proteins interaction with Cyclin D1, which has predicted the proteins which are involved in cell proliferation (Table 2; Fig. 5).

In the previous studies reported that, over-expression of CCND1 causes disruption of the normal cell cycle and DNA repair process of the cells, which leads to carcinogenesis (Sgambato et al., 2002; Kandel et al., 2001; Hemmer et al., 2001). In the another later study, reported that CCND1 found to be overexpressed in oral squamous cell carcinoma (Saawarn et al., 2012; Santarius et al., 2010; Michalides et al., 1995). Moreover, the association of individual cigarette smoking history and polymorphism of 870A allele of CCND1 has been found (Duan et al., 2013). As suggested in the previous research study CCND1 could be the potential target for cancer therapy (Saini et al., 2011; Weinstein et al., 2006). The relationship between CCND1 protein and its association with anti-oxidative levels in various cancers were well studied previously in which they confirmed that cyclin D1 expression is essential for cell proliferation and cancer cell survival (Laphanuwat et al., 2018).

5. Conclusion

It was seen that in all these cancer cases (OSCC) there was a lowering of anti-oxidant enzymes - leading to cellular and nuclear damage, which can be overcome by improving nutritional status of these patients. We have described the mechanisms of damage and risk factors which include alcohol and smoking. Monitoring the personal habits is necessary to combat the spread of various diseases. Higher intake of foods with functional attributes including high levels of antioxidants is one of the strategies in preventing oral cancers and based on the Insilico analysis CCND1/Cyclin D1 could be the potential therapeutic target as is found to overexpress in oral squamous cell carcinoma.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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