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Serum ceruloplasmin as cancer marker in oral pre-cancers and cancers

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Abstract:

BACKGROUND AND AIM: Oxidative stress leads to a compensatory increase in levels of serum ceruloplasmin in patients with such imbalances. Greater than normal serum ceruloplasmin levels are noticed in numerous cancers including the leukemias and Hodgkin's lymphoma. The purpose of the present study was to estimate and evaluate the efficacy of serum ceruloplasmin levels as a potential biomarker in the early detection of oral potentially malignant epithelial lesions (PMELs) including leukoplakia, oral submucous fibrosis (OSMF), and oral squamous cell carcinoma (OSCC) patients.

MATERIALS AND METHODS: The present observational study was conducted over a period of 2 years wherein 100 subjects aged between 18 to 60 years were divided into four groups with Group A consisting of 25 healthy controls, Group B and C with 25 patients each, clinically diagnosed with oral leukoplakia and OSMF and Group D with 25 patients clinically diagnosed and histopathologically proven OSCC. The patients were subjected to incisional biopsy after routine hematological investigation while the same sera samples were used for analysis of serum ceruloplasmin levels.

STATISTICAL ANALYSIS USED: Comparison of serum ceruloplasmin levels between the groups was performed using one way analysis of variance (one way ANOVA) test while $P < 0.05$ was considered statistically significant.

RESULTS: The mean serum ceruloplasmin levels were found to be 43.19 ± 1.90mg/dl in subjects of group A, 47.68 ± 1.51mg/dl in group B, 47.74 ± 1.45mg/dl in group C and 47.73 ± 0.74mg/dl in group D. Using one-way ANOVA, statistically significant variations were found in the values of mean serum ceruloplasmin levels in subjects of the four groups (F-value = 59.58, $P = 0.0001$).

CONCLUSIONS: The observations of the present study revealed that serum ceruloplasmin levels were found to be raised in all 3 study groups including oral leukoplakia, OSMF and OSCC as compared to the controls while the results were found to be statistically significant.

Keywords:

Biomarker, early detection, oral potentially malignant epithelial lesions, oral squamous cell carcinoma, Serum ceruloplasmin

Introduction

Oral potentially malignant epithelial lesions (PMELs) are defined as those lesions and/or, conditions of oral mucosa that show features of epithelial dysplasia but are not frank malignant lesions.[1] The term pre-cancerous lesion/condition has been discarded since not all these lesions/conditions turn into malignancies. Thus, these changes of the mucosa are referred to as PMELs.[2,3] The most common lesion among these PMELs is oral leukoplakia while the frequent sites affected are the buccal mucosa, gingiva (alveolar mucosa) and vermilion border of lip. Leukoplakia is a white patch or, plaque that cannot be characterized clinically or, histopathologically as any other disease.[4] The rate of malignant transformation of leukoplakia has been rated as around 0.13-34%.[5] Erythroplakia

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is nothing apart from an advanced stage or, variant of leukoplakia which has predominant red elements in it indicating even higher chances of dysplasia and being far more prone for malignant transformation.[4,5]

Likewise, oral submucous fibrosis (OSMF) is an insidious chronic disease process affecting any part of the oral mucosa and associated with juxta-epithelial inflammatory reaction followed by fibro-elastic changes in lamina propria with epithelial atrophy.[6] The condition is characterized by burning sensation of oral mucosa assisted with ulceration and pain, blanching of mucosa along with depapillation of tongue, depigmentation of mucosa and progressive reduction in mouth opening.[2] The overall prevalence of OSMF in India is estimated to be about 0.2%–0.5% and the prevalence is seen to vary with gender being 0.2%–2.3% in males while 1.2%–4.57% in females. It shows a high degree of malignant potential which ranges between 2.3% and 7.6%.[7]

Oral cancer is the most common malignancy known in the head and neck region and is one of the major causes of deaths worldwide. Approximately 80,000 new cases of oral cancers are diagnosed each year, mainly, due to consumption of different forms of tobacco products such as gutkha, quid, snuff or, misri.[8] Annually, 1,30,000 people succumb to oral cancers which translates into approximately 14 deaths per hour in India.[9] The most commonly encountered oral neoplasm is oral squamous cell carcinoma (OSCC) and it accounts for 95% of all oral cancers reported.[5] Squamous cell carcinoma has been defined by Pindborg and Sirsat[6] as a malignant epithelial neoplasm exhibiting squamous differentiation characterized by formation of keratin and/or, presence of intercellular bridges. All PMELs, eventually, progress to develop invasive OSCCs. To predict this aggressiveness, grading of the neoplasm is done based on assessment of the degree of keratinization, cellular, and nuclear pleomorphism and mitotic activity which help in the assessment of prognosis as well as deciding treatment guidelines for the disease.[5]

Despite recent advances in cancer treatments, the outcome and prognosis of OSCC is still poor. The lacuna for this lies in delayed and late diagnosis of neoplasm when the tumor is already in the advanced stages of disease.[10] An early enough diagnosis is, thus, highly warranted to initiate treatment in the initial stages itself to arrest progression of the malignant process. The stability, progress of PMELs into frank malignancies and/or, their regression, though, are not predictable by clinical and histological features and here, comes the role of tumor markers which are certain specific substances released either by tumor or, host, while combating the tumor, into the serum. The identification of such substances which can predict disease progression is, thus, of utmost importance in the management of these lesions.[11]

Classically, a marker is synthesized by tumor cells and released into circulation in large quantities during the process. Altered concentration of these biomarkers in the serum or, saliva of an individual, then, gives signal of the future alarming condition pertaining to the process of frank malignant transformations.[12] Ceruloplasmin exhibits a copper-dependent oxidase activity associated with the possible oxidation of ferrous (Fe2+) ions into ferric (Fe3+) ions, therefore, assisting in its transport in plasma in association with transferrin, capable of carrying iron only in ferric state.[13] Oxidative stress is an imbalance resulting out of free radical damage and disruption of oxidant-antioxidant balance in the body.[14] It has been observed that oxidative stress might lead to compensatory increased levels of serum ceruloplasmin in patients with such imbalances. Greater than normal serum ceruloplasmin levels are noticed in numerous cancers including the leukemias, Hodgkin's lymphoma as well as during copper toxicity or, zinc deficiency, acute and chronic inflammations, rheumatoid arthritis and secondary to drugs such as carbamazepine, phenobarbital, and valproic acid.[15,16] Serum ceruloplasmin levels are reduced in patients with hepatic disorders due to reduced synthesis, in Wilson's disease (copper storage disease), overdose of Vitamin C or, in aceruloplasminemia.[17] The purpose of the present study was to estimate and evaluate the efficacy of serum ceruloplasmin levels as a potential biomarker in the early detection of oral PMELs including oral leukoplakia, OSMF, and OSCC patients.

Materials and Methods

The present observational study was conducted over a period of 2 years wherein 100 subjects aged between 18 to 60 years clinically diagnosed and histopathologically confirmed with oral leukoplakia, OSMF and OSCC were divided into 4 groups with each group consisting of 25 patients as Group A consisting of 25 healthy controls, Group B with 25 patients clinically diagnosed with oral leukoplakia, Group C with 25 patients clinically diagnosed with OSMF and Group D with 25 patients clinically diagnosed and histopathologically proven OSCC. Subjects with present or, past history of any major illness such as liver disease, diabetes, hypertension, and tuberculosis, subjects undergoing radiotherapy or, chemotherapy for cancer, patients with a history of malignancy other than oral cancers and those who were >60 years of age were excluded because of possible immunocompromise. Biopsy was considered as the gold standard for confirmation of diagnosis. Ethical clearance was obtained from the Institutional Ethics Committee while the subjects were

informed in detail regarding the need and protocol of study and a written, informed consent was obtained from them. All the subjects, the patients and controls, were examined thoroughly [Figure 1] and a detailed history was recorded. The patients diagnosed with oral leukoplakia, OSMF and OSCC were, then, subjected to incisional biopsy taking tissue from the periphery of lesions along with adjacent normal tissue after routine hematological investigations. The same sera samples were, then, used for the analysis of serum ceruloplasmin levels in patients while sera was drawn from the controls and similar procedure was followed for analysis of serum ceruloplasmin levels in both patients and controls using human ceruloplasmin elisa kit manufactured by cusabio assay max [Figure 2].

Statistical Analysis Used: The statistical analysis was done using the Statistical Package for Social Sciences [SPSS version 17.0 (SPSS Inc., Chicago, IL, USA), Epi-Info 6.0 version] and Graph Pad Prism version 5.0. Comparison of serum ceruloplasmin levels between groups was performed using one way analysis of variance (one-way ANOVA) test (F-Test) while frequencies were compared with the help of Chi-square test. Inter-group comparisons and multiple comparisons were done by Tukey's test. P <0.05 was considered statistically significant.

Results

Table 1 reveals age-wise distribution of patients wherein statistically significant differences were observed in the mean age of patients in 4 groups on using Chi-square test ($\aleph^2$-value = 69.50, P = 0.0001). Likewise, Table 2 reveals gender-wise distribution of patients wherein using Chi-square analysis, statistically significant differences were found as far as gender of patients was concerned in all 4 groups ($\aleph^2$-value = 8.94, P = 0.030). Table 3 reveals habit-wise distribution of patients showing 16% of subjects in group A, 68% in group B, 96% in group C and 36% in group D were having a history of gutkha chewing while 4% of subjects in Group A and C each and 8% in Group D had history of betel nut chewing. Furthermore, 4% of subjects in group A and 32% of subjects each in Group B and Group D had history of smoking while 24% of subjects in group D had history of alcohol consumption. Furthermore, 76% of subjects in group A had no such deleterious habit. Analyzing the findings using Chi-square test revealed statistically significant differences in relation to habit-wise distribution of subjects as well in all 4 groups ($\aleph^2$-value = 107.60, P = 0.0001). Table 4 reveals the descriptive statistics comparing mean serum ceruloplasmin levels in the 4 groups with the mean serum ceruloplasmin levels to be 43.19 ± 1.90mg/dl in subjects of Group A, 47.68 ± 1.51mg/dl in group B, 47.74 ± 1.45mg/dl in Group C and 47.73 ± 0.74mg/dl in group D. Using one-way ANOVA, statistically significant variations were found in the values of mean serum ceruloplasmin levels in subjects of the 4 groups (F-value = 59.58, P = 0.0001).

Discussion

Cancer, a disorder of cellular behavior, is characterized by alteration of serum glycoproteins and cell surface glycosylation and is associated with various types of transformation processes. Early detection and early treatment of oral PMELs and oral cancers not only reduces mortality, but, also, renders quality life to the survivors.

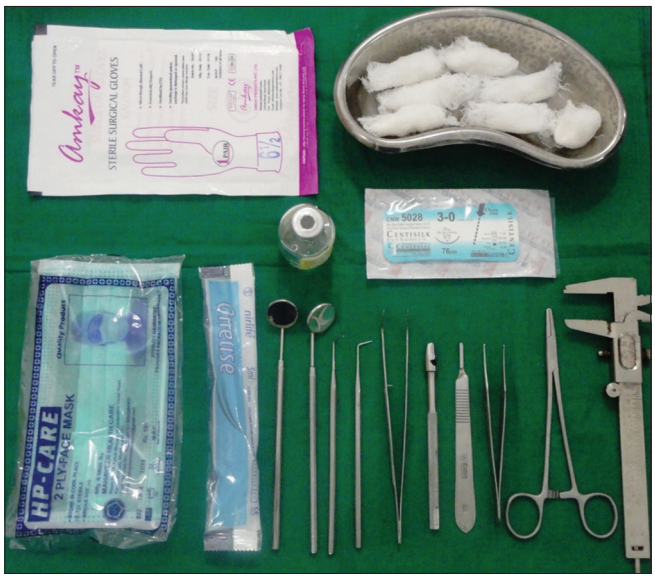

Figure 1: Armamentarium for clinical examination

Figure 2: Human ceruloplasmin enzyme-linked immuno-sorbent assay kit (assay max) for estimation of serum ceruloplasmin levels

Table 1: Age-wise distribution of patients

Age group (years)	Group A, *n* (%)	Group B, *n* (%)	Group C, *n* (%)	Group D, *n* (%)	χ^2, *P*
20-29	16 (64)	2 (8)	9 (36)	0	69.50, 0.0001*
30-39	8 (32)	6 (24)	5 (20)	1 (4)	
40-49	1 (4)	13 (52)	7 (28)	6 (24)	
50-59	0	4 (16)	4 (16)	16 (64)	
≥60	0	0	0	2 (8)	
Total	25 (100)	25 (100)	25 (100)	25 (100)	
Mean±SD	28.36±5.79	41.32±8.02	36.52±10.27	53.0±5.46	
Range	20-40	28-55	22-55	39-60	

*P<0.05 statistically significant. SD: Standard deviation

Table 2: Gender-wise distribution of patients

Gender	Group A, *n* (%)	Group B, *n* (%)	Group C, *n* (%)	Group D, *n* (%)	χ^2, *P*
Male	16 (64)	24 (96)	21 (84)	21 (84)	8.94, 0.030*
Female	9 (36)	1 (4)	4 (16)	4 (16)	
Total	25 (100)	25 (100)	25 (100)	25 (100)	

*P<0.05 statistically significant

Table 3: Habit-wise distribution of patients

Habit	Group A, *n* (%)	Group B, *n* (%)	Group C, *n* (%)	Group D, *n* (%)	χ^2, *P*
Gutkha	4 (16)	17 (68)	24 (96)	9 (36)	107.60, 0.0001*
Betel nut	1 (4)	0	1 (4)	2 (8)	
Smoking	1 (4)	8 (32)	0 (0)	8 (32)	
Alcohol	0	0	0	6 (24)	
No habit	19 (76)	0	0	0	
Total	25 (100)	25 (100)	25 (100)	25 (100)	

*P<0.05 statistically significant

Table 4: Descriptive statistics revealing serum ceruloplasmin levels in various groups

Group	*n*	Mean	SD	SE	Minimum–maximum
Group A	25	43.19	1.90	0.38	40.00-45.90
Group B	25	47.68	1.51	0.30	42.00-49.50
Group C	25	47.74	1.45	0.29	44.70-49.70
Group D	25	47.73	0.74	0.14	46.10-48.90

One-way ANOVA

Source of variation	Sum of squares	df	Mean square	*F*	*P*
Between groups	383.58	3	127.86	59.58	0.0001*
Within groups	206.006	96	2.14		
Total	589.58	99			

Multiple comparisons: Tukey's test

Group	Mean difference (I–J)	SE	*P*	95% CI (lower bound–upper bound)
Group A				
Group B	4.48	0.41	0.0001*	3.40-5.56
Group C	4.54	0.41	0.0001*	3.46-5.63
Group D	4.53	0.41	0.0001*	3.45-5.61
Group B				
Group C	0.06	0.41	0.999	1.01-1.14
Group D	0.05	0.41	0.999	1.03-1.13
Group C				
Group D	0.01	0.41	1.000	−1.09-1.07

*P<0.05 statistically significant. SD: Standard deviation, SE: Standard error, CI: Confidence interval, ANOVA: Analysis of variance

A majority of neoplasms are preventable as well as curable if they are detected in the early enough stages, especially, oral PMELs like oral leukoplakia and OSMF which usually precede frank oral cancers.[18] Biomarkers provide a noninvasive means of diagnosis, facilitate early detection of PMELs or, malignant conditions and their early treatment eventually affects the prognosis these lesions have after treatment. Cell membrane mainly consists of glycoproteins and glycolipids. Glycoproteins are protein-carbohydrate complexes in

which oligosaccharides and or, polysaccharides are joined to specific amino acids of proteins by covalent linkages.[18] Till now, many researchers have developed and successfully demonstrated the use of protein markers as biomarkers in the diagnosis and management of oral cancers. The present study evaluated the diagnostic utility of serum ceruloplasmin as a potential cancer marker.

The age of subjects in the control group (A), patients with oral leukoplakia (B), OSMF (C) and OSCC (D) ranged from 20-40 years, 28-55 years, 22-55 years, and 39-60 years respectively in the present study, thus, showcasing a broad range of probability of the occurrence of oral PMELs and OSCC in the affected patients. From the analysis of data, it was evident that OSCC showed progression with ageing and became evident in the 5th decade of life. This observation was in accordance with the studies conducted by Chittemsetti *et al*.[19] and Shetty *et al*.[20]. There are varying reports on sex ratio in different published studies. In the present study, out of 25 oral leukoplakia patients, 24 (96%), 25 OSMF patients, 21 (84%), 25 OSCC patients, 21 (84%) of the patients were male while 1 (4%), 4 (16%), and 4 (16%) of the patients were females respectively indicating a male predominance. The male to female ratio in the OSCC group in the present study was found to be 5.25:1 which was similar to the findings of the study conducted by Shetty *et al*.[20] who found a higher male prevalence reported for the lesions with a male to female ratio of 5:1. In other studies by Elango *et al*.[21] and Mehrotra and Yadav,[22] the male to female ratio was found to be 4:1 while the mean age of patients being 55.92 ± 10.17 years.

The occurrence of oral PMELs and oral cancers is seen to be higher in the males and this might be due to the much prevalent habit of chewing gutkha, betel nut, smoking, and drinking alcohol in males as compared to females. Literature is abuzz with correlation of oral malignancies and habits such as smoking and tobacco chewing. Gutkha chewing (36%) and smoking (32%) were the most common habits found in the present study followed by alcohol (24%). Thus, gutkha chewing, smoking and alcohol were found to be the major risk factors in the present study. Similar risk factors in head and neck malignancies have been reported in various other studies including the ones conducted by Shashikanth and Rao[23] and Day and Blot.[24]

Ceruloplasmin is a glycoprotein encoded by CP gene on chromosome no. 3q24 involved in the transport of copper ions in body and also, involved in iron metabolism by virtue of ferroxidase activity. It is synthesized primarily in the liver and contains 6-7 copper ions. Ceruloplasmin is an acute phase reactant and a transport protein.[16] A balance between oxidant carcinogens and endogenous antioxidant defenses is of particular relevance to the process leading to the evolution of carcinogenesis. Oxidative stress is an imbalance between free radical damage and the antioxidant protection in body. Copper and ceruloplasmin have been observed to be significantly increased in numerous cancers. Cupric ions are reported to inhibit the production of singlet oxygen and this is of particular significance because of the latter's ability to cross the cell membrane and its high reactivity toward various biomolecules.[25-29]

Ceruloplasmin levels, as assessed in the present study, showed significantly higher levels in all 3 study groups including oral leukoplakia (B), OSMF (C) and OSCC (D) with the corresponding values being 47.68 ± 1.51mg/dl, 47.74 ± 1.45mg/dl and 47.73 ± 0.74mg/dl respectively as compared to the controls (A), 43.19 ± 1.90mg/dl. The results of the present study suggested that as compared to the controls (A), ceruloplasmin levels remained significantly high ($P < 0.05$) in all 3 study groups including oral leukoplakia (B), OSMF (C) and OSCC (D), though, were found to be nearly constant. A better correlation could be observed between elevated serum copper levels and some malignant tumors as in the studies conducted by Mailer *et al*.[30] and Linder *et al*.[31] This was probably the first evidence which created ceruloplasmin as a potential candidate to be used as a biomarker for oral cancers. Similar correlation was found between increased copper levels and areca nut chewing habit in the study conducted by Arakeri *et al*.[32]

In the present study, it was observed that controls (A) revealed serum ceruloplasmin levels to be 43.19 ± 1.90mg/dl which was found to be slightly on the higher side when compared to an earlier reported study conducted by Senra Varela *et al*.[33] who found the mean serum ceruloplasmin levels in the controls to be 32.4mg/dl. In the present study, the mean serum ceruloplasmin levels were significantly higher ($P < 0.05$) in the OSCC (D) group as compared to the controls, the level of change, though, with respect to the other PMELs, oral leukoplakia (B) and OSMF (C) and OSCC (D), did not represent any significant variation.

Jayadeep *et al*.,[34] also, reported similar findings in relation to serum ceruloplasmin levels in their study on oral pre-cancerous lesions and frank oral cancers highlighting not only the diagnostic significance serum ceruloplasmin levels have in the early detection of malignancies but prognostic significance marking disease progression. The increased serum ceruloplasmin levels indicate elevated antioxidant activity of the serum. Serum ceruloplasmin, apart from being an effective antioxidant protein, is one of the acute phase reactants, the concentration of which increases in the plasma after tissue injury. The acute phase reactants protect the tissue

as a whole from the deleterious effects caused due to the release of free radicals and oxidation products, thus, suggesting that the body responds to free radical damage by raising the antioxidant activity of plasma by elevated ceruloplasmin levels.[35]

Decreased levels of copper are of great significance in the etiology of OSMF as has been reported in numerous other studies including the ones conducted by Jani *et al*.[36] and Patil and Joshi[37] to name a few. The decrease in copper levels obviously brings about a decrease in serum ceruloplasmin levels, thus, influencing iron absorption and mobilization of iron from the liver and other tissue stores.[38] Further deficiency in copper levels, also, affects iron absorption and thus, leads to iron deficiency.

In accordance with the findings of the present study, Akinmoladun *et al*.,[39] also, found increased mean serum ceruloplasmin levels in patients with oral PMELs and frank oral cancers in their study. Increased serum ceruloplasmin levels have, also, been previously reported in various malignancies.[40,41] The reasons for the same, though, not clearly understood, could be due to serum ceruloplasmin being an acute phase reactant apart from being a direct response to increase in copper levels, which, also, had, been similarly reported in various malignancies.[25-29]

Even though not being sensitive for phase base reactions, usefulness of serum ceruloplasmin levels never becomes less as could be seen from the findings of the present study where its sensitivity and specificity had been benchmarked in various oral PMELs and frank OSCC and it is noteworthy that it is expressed by the tumoral cells just as the other established biomarkers and hence, makes it a featured marker of future use in such situations when investigated in detail as, also, concluded by Kunapuli *et al*.[42] and Abd-el-Fattah *et al*.[43]. Andrzejewska *et al*.,[44] also reported the said correlation between serum ceruloplasmin levels and various clinical stages of cancer of larynx as, also, in assessing prognosis in such cases as was observed by Krecicki and Leluk[45] who concluded that the determination of serum ceruloplasmin levels could be of use in monitoring of cancer patients.

There are few possible limitations that need to be addressed as far as the present study is concerned. First, the serum ceruloplasmin levels were not correlated with the histopathological grading and staging of the included oral PMELs including oral leukoplakia, OSMF and OSCC. Second, serum ceruloplasmin levels were significantly higher in the oral PMELs as compared to the controls, though, inter-group variations were reported to be nearly constant in all 3 study groups. Third, increase in serum ceruloplasmin levels in humans is seen in chronic inflammatory processes, active hepatitis, biliary liver cirrhosis, and malignant tumors. It becomes imperative, therefore, to detect the correct cause of increase in serum ceruloplasmin levels and thus, render a need to pre-evaluate the hepatic and renal diseases in future research projects. Furthermore, post-treatment serum ceruloplasmin levels were not evaluated, so, their impact on the prognosis of the lesions cannot be commented upon. Serum ceruloplasmin, being in the very primitive stages, thus, needs more researches to contribute as a specific biomarker for oral cancers.

Conclusions

The observations of the present study revealed that serum ceruloplasmin levels were found to be raised in all 3 study groups including oral leukoplakia, OSMF and OSCC as compared to the controls while the results were found to be statistically significant. In addition, a definitive association was found between harmful habits in decreasing order of gutkha chewing, smoking, alcohol consumption, and betel nut to the incidence of oral PMELs and frank OSCC. Thus, it could be concluded from the observations of the present study that serum ceruloplasmin in conjunction with clinical diagnostic procedures can be used as a potentially reliable, adjunctive serological marker for monitoring and assessing oral PMELs and frank OSCC.

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Conflicts of interest
There are no conflicts of interest.

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