A Study of Rhodamine-6G Stain Expression for Detection and Association of Copper Content in Potentially Malignant Disorders and Squamous Cell Carcinoma

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Abstract

Aim and Objective: To assess the expression of Rhodamine-6G stain in potentially malignant disorders and SCC and to determine association of copper in potentially malignant disorders and OSCC.

Materials and Method: Sections from ten tissue blocks of each normal oral mucosa, oral leukoplakia, oral sub mucous fibrosis (OSMF), well differentiated squamous cell carcinoma (WDSCC) and moderately differentiated squamous cell carcinoma (MDSCC) was taken and stained with Rhodamine-6g stain. After adequate staining, all the samples were assessed using Fluorescence microscope for the color intensity and depth of penetration of copper and compared.

Results: It showed significant Pearson chi square values of 0.157 for color intensity of copper and 0.019 for depth of penetration of copper. Pearson correlation was calculated which showed significant value for leukoplakia (0.055)* in comparison to OSMF (0.429), WDSCC (-.395) and MDSCC (0.372).

Conclusions: Increase levels of copper in leukoplakia would be because of the fact that copper helps in activating many angiogenic factors which further helps in tumor progression from premalignant to malignancy.

Keywords: *Rhodamine-6G, Leukoplakia, Oral Sub mucous Fibrosis, Squamous cell carcinoma, Copper content, Fluorescent Microscopy.*

Introduction

Trace elements are important constituents of biological enzyme systems. They are found to play a central role in a various physiological metabolic process and in the pathology of various diseases of these elements, copper has been studied more extensively for its role in various bodily processes.[1,2] In absence or deficiency of copper, various enzymes in the body are not be able to perform their metabolic functions, as they require metallic ions such as copper to get activated. For example, ceruloplasmin, tyrosinase and cytochrome oxidase. These enzymes play an essential role in various redox reactions, free radical formation and maintenance of cellular protein homeostasis.[3,4] A number of angiogenic factors, for example TNF-α, IL-1 and VEGF are activated by copper which on further binding to endothelial cells result in their proliferation. During the progression of the cancerous lesion, copper level is reported to be unbalanced.[5]

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Occurrence of cancer, precancer and other tumor-like lesions in oral cavity is very high now-a-days in the developing countries.[6] The reason for the high incidence is not fully known, however it may be due to various contributing factors like smoking, chewing tobacco, sharp impinging prosthesis among others.

In existing literature, serology and whole blood analysis for tracer elements has been correlated with various diseases and their pathogenesis. Very few studies have been conducted using the tissue sections with respect to potentially malignant disorders and OSCC. Therefore, a retrospective study was conducted to analyze the presence or absence of copper content in potentially malignant as well as in malignant lesions using Rhodamine-6G stain and also to evaluate its significance as a diagnostic tool in disease progression.

Materials and Method

The current study was carried out in the Department of Oral Pathology, after taking the approval from Institutional Research and ethical Committee at M.M. College of Dental Sciences & Research, M.M. (Deemed to be University), Mullana.

Formalin fixed and paraffin embedded blocks of previously diagnosed cases were obtained from archives of the department. Normal healthy gingival tissues were obtained from the excess gingival tissue which were excised and discarded during periodontal flap surgery and extraction after taking the informed consent from the patients. The study groups were divided into three:

Group A: Control Group [Normal oral mucosa (n=10)]

Group B: Potentially Malignant Disorders (PMD)

- Leukoplakia (n=10)
- Oral sub mucous fibrosis (n=10)

Group C: Carcinoma

- Well-differentiated SCC (n=10)
- Moderately-differentiated SCC (n=10)

The slides were prepared by sectioning the paraffin embedded tissues with the help of manual rotary microtome. Tissues sections were than subjected to Rhodamine-6G staining procedure. They were assessed using Nikon Research (Eclipse 80i) fluorescent microscope and compared based on quantitative expression in various study groups.

Results

The criteria were set for the color intensity and depth of penetration as mild, moderate and severe. Analysis of color intensity and depth of penetration of copper was done in oral leukoplakia, OSMF, Well Differentiated SCC and Moderately Differentiated SCC. In the criteria for color intensity, out of 10 cases of oral leukoplakia, mild and moderate intensity were shown by 04 cases each, whereas severe intensity were seen in 02 cases. Out of 10 cases of oral sub mucous fibrosis, mild color intensity was seen in 07 cases, moderate color intensity was seen in 03 cases, whereas no case showed severe color intensity. In Well differentiated Squamous cell carcinoma cases, mild color intensity was seen in 02 cases, moderate color intensity was seen in 06 cases and only 02 cases showed severe color intensity. In Moderately differentiated Squamous cell carcinoma, out of 10 cases, mild color intensity was seen in 07 cases, moderate color intensity was seen in 01 case and 02 cases showed severe color intensity.

In the criteria for depth of penetration of copper, out of 10 cases of oral leukoplakia, no case showed mild depth of penetration, whereas moderate depth of penetration were seen in 04 cases and severe depth of penetration were seen in 06 cases. In oral sub mucous fibrosis, out of 10 cases, mild depth of penetration were seen in 03 cases, no case showed moderate depth of penetration, whereas 07 cases showed severe depth of penetration. In well differentiated squamous cell carcinoma, out of 10 cases, no case showed mild depth of penetration, moderate depth of penetration was seen in 02 cases and 08 cases showed severe depth of penetration. In Moderately differentiated Squamous cell carcinoma, out of 10 samples, no cases showed mild depth of penetration, whereas 05 cases each showed moderate and severe depth of penetration.

Categorical data was collected and chi square test was done for both color intensity and depth of penetration for the study samples. All the normal healthy tissues showed the absence of copper content (Figure 1). Tissue samples of oral leukoplakia (Figure 2), oral sub mucous fibrosis (Figure 3), Well-differentiated squamous cell carcinoma (Figure 4) and Moderately-differentiated squamous cell carcinoma (Figure 5) showed the presence of copper in epithelium and connective tissue. The test showed significant Pearson chi square values of 0.157 for color intensity of copper and 0.019 for depth of penetration of copper. Pearson correlation was done which showed

significant value for leukoplakia (0.055)* in comparison to OSMF (0.429), well differentiated SCC (-.395) and moderately differentiated SCC (0.372).

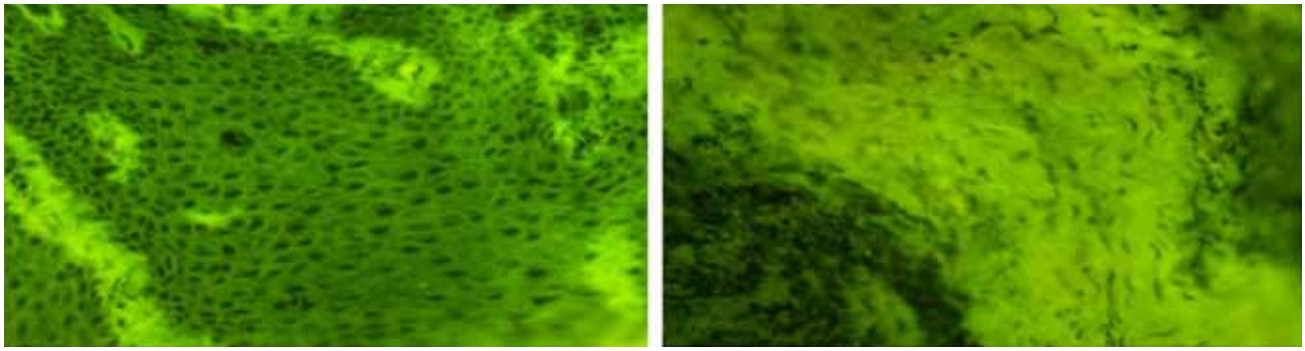

Figure 1: Absence of copper in both epithelium and connective tissue of the normal oral mucosa

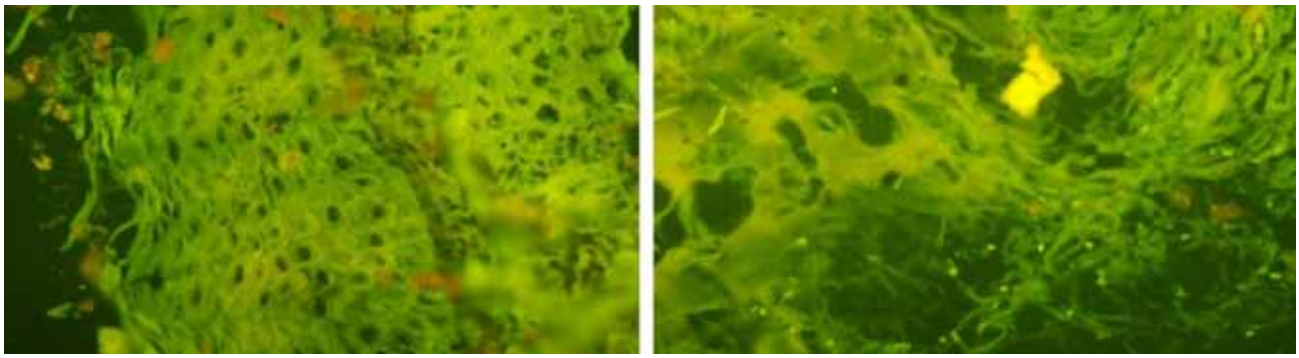

Figure 2: Presence of copper in epithelium and connective tissue in cases of leukoplakia

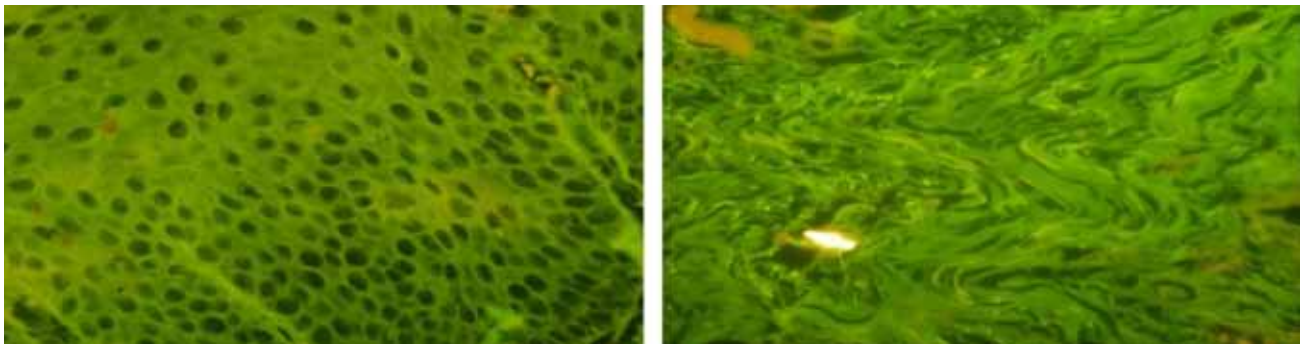

Figure 3: Presence of copper in epithelium and connective tissue of OSMF

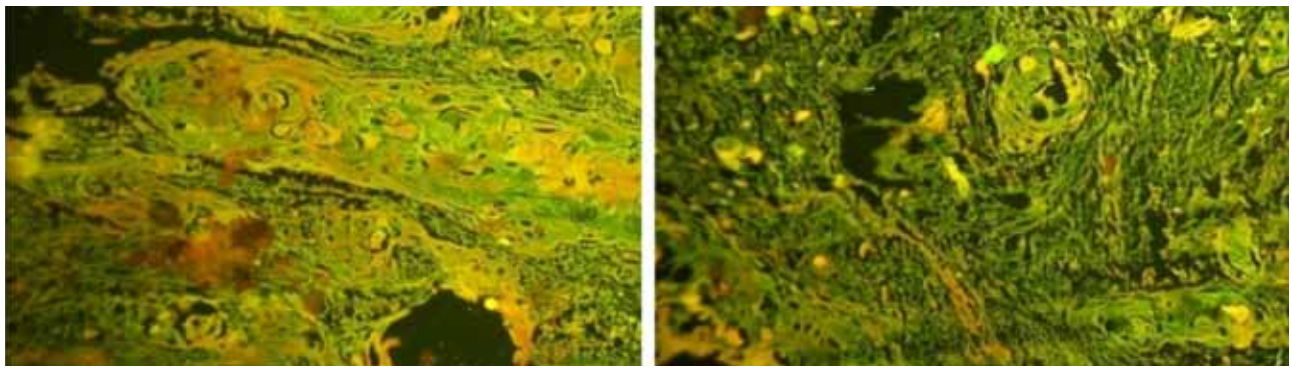

Figure 4: Presence of copper in epithelium and connective tissue of well differentiated squamous cell Carcinoma

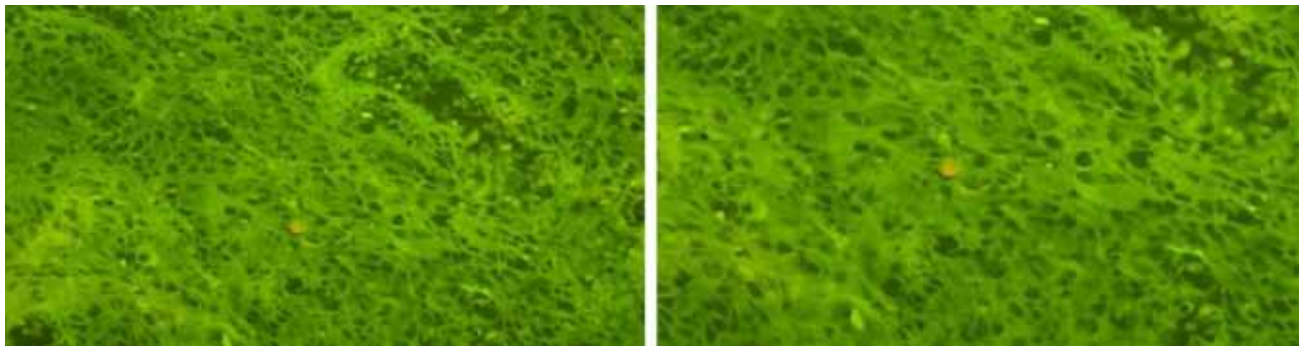

Figure 5: Presence of copper in epithelium and connective tissue of moderately differentiated squamous cell carcinoma

Discussion

Trace elements are an important constituents of biological enzyme systems. They are found to play a key role in numerous physiological metabolic processes and also in various pathological diseases. Among these trace elements, copper has been studied more extensively for its role in various bodily processes.[1,2]

In the absence or deficiency of copper various enzymes in the body are not be able to perform their metabolic functions, as they require them to get activated. For example, ceruloplasmin, tyrosinase and cytochrome oxidase. These enzymes play an essential role in various redox reactions, free radical formation and maintenance of cellular protein homeostasis.[3,4]

The present study was aimed at assessing the expression of Rhodamine-6G stain under the fluorescent microscope in potentially malignant disorders and SCC and to determine its association with copper. A total of 40 study samples were taken for the study from the archival blocks from the department of Oral Pathology. For comparison, 10 normal healthy gingival tissues were obtained after taking the informed consent from the patients, from excess gingival tissue excised and discarded during periodontal flap surgery and extraction.

Criteria for scoring the color intensity and depth of penetration were set. For color intensity, criteria were set as mild, moderate and severe and the color intensity of copper in cases of oral Leukoplakia, OSMF and squamous cell carcinoma were observed.

Categorical data was collected and the Pearson chi square test was done for both color intensity and depth of penetration for the study samples. All the normal healthy tissues showed the absence of copper content. The test showed significant Pearson chi square values of 0.157 for color intensity of copper and 0.019 for depth of penetration of copper. Pearson correlation was calculated which showed significant value for leukoplakia (0.055)* in comparison to OSMF (0.429), WDSCC (-.395) and MDSCC (0.372).

A study done by Swain N and Ray JG found that copper content was increasingly higher from leukoplakia to cancer group.[7] Ayinampudi BK and Narsimhan M, observed significant increase in salivary copper levels in leukoplakia.[8] Similar findings were observed in our study with Rhodamine 6G staining technique on paraffin embedded tissue sections in comparison to the latter.

Toke et.al in their study found increased levels of serum copper as well as serum zinc in malignant patients as compared to the potentially malignant disorders.[9] Their findings were different to what is seen in our study.

Study done by Shetty SR et.al observed significant raise in the serum copper values in OSMF patients in contrast to controls.[10] According to Joshi PS et al. higher mean values for copper was seen in cases of oral sub mucous fibrosis (2.00) in comparison to leukoplakia and squamous cell carcinoma.[11] Our findings in the present study were different in comparison with the above study which showed no significance values for OSMF and SCC but rather showed highly significant values for oral leukoplakia.

Khanna SS and Karjodkar FR in their study found mean serum copper values were notably high in patients sera with premalignant as well as malignant lesions. In comparison, in our study only premalignant lesion (leukoplakia) showed significant values for copper.[12]

Ehud J et.al revealed that copper levels were raised in malignancy in comparison to zinc whose levels were found to be decreased.[13] Their findings were similar to the findings in the present study in relation to the depth of penetration of copper which showed increase levels in SCC.

Irons RD et.al[14] in their study revealed the presence of abnormal tissue copper levels in histopathological sections of liver using the Rhodamine staining technique, whose findings were similar to the present study. Varghese I et.al[15] found significant reduction in levels of copper in OSMF and SCC in comparison to leukoplakia which was similar to the present findings.

Conclusion

Increase levels of copper in leukoplakia would be because of the fact that copper helps in activating many angiogenic factors which further helps in tumor progression from premalignant to malignancy. During this progression copper level is reported to be increased and unbalanced. Since the chances of oral leukoplakia progressing to cancer is more as compared to other potentially malignant disorders, our study has showed significant values for copper in leukoplakia as compared to OSMF and SCC.

Regular follow up of all the potentially malignant lesions with periodic estimation for copper content using Rhodamine 6G staining technique as potential marker in assessing the disease progression should be helpful in correlating the progression from potentially malignant stage to malignancy.

Ethical Clearance: Ethical clearance for the present study was taken from the Institutional Ethical Committee.

Sources of Funding: Nil

Conflicts of Interest: None

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