EXPRESSION OF LORICRIN IN ORAL SUBMUCOUS FIBROSIS, HYPERKERATOSIS AND NORMAL MUCOSA: AN IMMUNOHISTOCHEMICAL STUDY

Dissertation submitted to

THE TAMILNADU Dr.M.G.R.MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

APRIL 2013

CERTIFICATE

This is to certify that this dissertation titled **"EXPRESSION OF LORICRIN IN ORAL SUBMUCOUS FIBROSIS, HYPERKERATOSIS AND NORMAL MUCOSA: AN IMMUNOHISTOCHEMICAL STUDY"** is a bonafide dissertation performed by **NITHYA.S** under our guidance during the postgraduate period 2010-2013.

This dissertation is submitted to **THE TAMILNADU DR. M.G.R MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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Acknowledgement

It is not every day that we get to thank all those who really matter to us, and acknowledging this brings joy and pride so deep that it makes me feel whole and cherished .I would want to thank God foremost for his benevolence and love for making it possible, for every breath, every thought and every walk of life is filled with his goodness

I would like to express my deep gratitude and reverence to my post graduate teacher and mentor ***Dr. K. Ranganathan****, MDS, MS (Ohio), Ph.D, Professor and Head, Department of Oral and maxillofacial pathology, Ragas Dental College and Hospital for his valuable guidance, constant support and encouragement throughout my dissertation. His penchant for perfection in all things had been a beacon guiding us in our study.*

My sincere thanks to ***Dr. Umadevi K. Rao****, Professor, Department of Oral and maxillofacial pathology Ragas Dental College and Hospital for her constant support, motivation to learn and encouragement in times of trials throughout my study.*

I extend my heartfelt gratitude to my Guide and Professor ***Dr. Elizabeth Joshua****, Department of Oral and maxillofacial pathology Ragas Dental College and Hospital for her constant guidance, support, tons of patience and endearing words of advice to explore all aspects in the completion of my work.*

I earnestly thank Professor, ***Dr. T. Rooban****, Department of Oral and maxillofacial pathology Ragas Dental College and Hospital for his encouragement and concern in helping me to complete this study*

*My sincere thanks to the principal **Dr. Ramachandran S** and Chairman, **Mr. Kanagaraj A**, Ragas Dental College and Hospital for their permission to use the facilities of the institution.*

*I also thank **Dr. K.M. Vidya** Reader, **Dr. N. Lavanya** Reader, **Dr. C. Lavanya** Senior Lecturer, Department of Oral and Maxillofacial Pathology Ragas Dental College and Hospital for their constant motivation and support throughout my study.*

*I am deeply indebted to **Dr.Rajkumar** Professor and HOD of SRM dental college and hospitals Chennai , **Dr.Shahela Tanveer** senior lecturer, Sri Sai Dental College, Vikarabad Andhrapradesh for granting me permission and giving me access to their archival samples for my study. I would also like to specially thank my uncle and friend **Dr. P.K Palaniraj M.D Paediatrics.** for helping me to procure my control sample.*

*I am very grateful to our Research Assistant **Mrs. Kavitha Wilson,** and Lab Technician **Mr.Rajan**, Department of Oral and Maxillofacial Pathology Ragas Dental College and Hospital who with their timely inputs smoothed away the difficulties in the course of my dissertation.*

I also owe my thanks to ***Dr. Ravanan D Ramanujam*** *Professor, Department of statistics Presidency college Chennai for his statistical help in my dissertation.*

I acknowledge gratefully the help of my friends and batch mates ***Dr.Aiswarya Lekshmy S. U, Dr. Jaish Lal M.S, Dr.Shanmugapriya P, Dr.Sudharsan R and Dr. Vaishnavi S*** *who by their very presence and solidarity made this endeavour a pleasant experience****.*** *I also thank my* ***Seniors and Juniors*** *for their support and encouragement.*

I specially would love to thank my loving husband, ***Mr. R. Prabakar B.E, M.S, M.B.A*** *whose unstinting love, sacrifice and encouragement has made me realize the true meaning of life, my children* ***P.N.Shakthi Vananth*** *and* ***P.N.Kavin Meenakshi*** *who complement my existence, my dear mother in law* ***Mrs. Suseela Rajagopal,*** *my beloved mother* ***Dr.Pankajakshi B.S..C, M.B.B S*** *who is my inspiration, for never having lost her faith in me and my sister* ***Vidhya*** *for always being there for me.*

They had all been a driving force with their love, understanding, support, patience and prayers in this endeavour of mine.

Above all I sincerely thank my ***Patients*** *without whom this study would not have been completed.*

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ABSTRACT

Back ground

Loricrin is a late differentiation protein of terminally differentiated keratinocytes seen in the cornified envelope. It is seen in abundance in the keratinizing epithelium, which are subjected to considerable mechanical stress. The coarse fibres of areca nut and the continuous friction from occluding teeth are the major causes of mechanical stress to the oral mucosa in oral submucous fibrosis and hyperkeratosis respectively. This study tries to relate the expression of loricrin in these two lesions to the habit of chewing and also its role as a potential marker to diagnose these conditions..

Aim and objectives. To evaluate the expression of Loricrin in the epithelium of formalin fixed, paraffin embedded tissue specimens of oral submucous fibrosis, hyperkeratosis and normal mucosa by immuno histochemistry.

Materials and methods. Archival samples of tissue specimens (n=73), which were formalin fixed and paraffin embedded were stained with antibodies to loricrin and the intensity of staining was graded as (-) negative, (+) mild,(++)moderate and (+++) intense. Pearson's chi square test was done for statistical analysis.

Results and Conclusion. Loricrin expression was observed in all the groups with prominent staining in the stratum granulosum and had a significant association to habits.(P=0.000).The prominent staining indicates a compensatory mechanism in the surface of the epithelium in the early stages of oral submucous fibrosis and has the potential to be an early marker of the condition.

Key words - Loricrin, OSF, Areca nut Hyperkeratosis.

Introduction

Oral carcinoma is one of the most common cancers in India and proceeds through a stepwise accumulation of genetic damage over time[1]. The oral cavity is easy to examine and there is a great opportunity to improve patient outcomes through diagnosis and treatment of oral potentially malignant disorders before the development of invasive oral carcinoma. Developments in detection and diagnosis of oral potentially malignant lesions and innovative approaches in management of early oral neoplasia by means of technological and therapeutic advances are much needed to improve the poor outcomes associated with oral cancer. Genetic mutations often produce early phenotypic changes that may present as clinically apparent, recognizable lesions. Clinical presentations of the oral cavity that are recognized as potentially malignant were classified into two broad groups, taking into account the diverse clinical appearances presented world wide as lesions and conditions.[1]

A **potentially malignant lesion** is a morphologically altered tissue in which oral cancer is more likely to occur than in its apparently normal counterpart or a generalized state associated with a significantly increased risk of Cancer. This was proposed by an earlier working group of the world health organization (WHO) in 1978. [2]

The histological finding of epithelial dysplasia is strongly associated with an increased rate of invasive cancer development. Some molecular biomarkers with potential diagnostic relevance include DNA content and chromosome polysomy, loss of heterozygosity (LOH), nucleolar organizer regions, histo-blood group antigens, proliferation markers such as Ki-67, increased Epidermal growth factor receptor (EGFR), and decreased expression of p16, and p53. Although a

reliable, validated marker panel for providing clinically useful prognostic information in OPL patients has not yet been established, the advent of genomic and proteomic analysis techniques may soon yield major advances toward a prognostically relevant molecular classification system [2,3]

Certain proteins can act as potential markers in determining the various stages of these potential malignant disorders and will serve as a valuable tool in providing a better outcome in both prognosis and treatment to the patient, one such marker could be Loricrin, which is a major protein of the cornified envelope of human epidermis, and is one of the differentiation markers of terminally differentiated keratinocytes. Its abundant presence in keratinizing epithelium, subjected to considerable mechanical stress has led to the assumption that expression of Loricrin is essential for the function of these tissues**.**

Oral submucous fibrosis is a chronic insidious disease affecting any part of the oral cavity and sometimes the pharynx, Occasionally preceded by and/or associated with vesicle formation and is always associated with a juxta-epithelial inflammatory reaction followed by progressive hyalinization of the lamina propria.[4]

This lesion represents 80% of potentially malignant oral lesions and is defined as a white patch or plaque that cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent except the use of tobacco. This is a diagnosis by exclusion for a lesion that cannot be given another specific diagnostic name and does not typically disappear with removal of known aetiological factors, excepting smoked tobacco.[5] Analysis using the microarray technique of the expression

profiles of 8,800 genes in human oral leukoplakia (n=4) and oral squamous cell carcinoma (OSCC) (n=2) was done to identify the genes responsible for its pathogenesis and malignant transformation. Eight genes were up-regulated (>2.0-fold) and ten were down-regulated (<0.5-fold) in all leukoplakia samples. Loricrin and keratins displayed greater differences between normal tissue and leukoplakia suggesting that gene abnormalities in cytoskeleton network components might be responsible for the development and progression of oral leukoplakia.[6,7]

This study is undertaken to demonstrate expression of loricrin (a key factor in maintaining the barrier function of the epidermis in keratinising epithelia), in non keratinising epithelia of oral mucosa attributing its presence to the mechanical stress induced by the chewing habits of areca nut in conditions like OSF and hyperkeratosis ,where its presence kicks in as a protective compensatory mechanism. The expression of this unique protein has been analysed in certain conditions with hyperkeratosis like frictional keratosis and epithelial dysplasia and oral submucous fibrosis (OSF) in this study

Aims and Objectives

AIM:

To evaluate the expression of Loricrin in the epithelium of Oral submucous fibrosis and hyperkeratosis and normal mucosa.

OBJECTIVES:

- To study the expression of loricrin in formalin fixed paraffin embedded tissue specimens of oral submucous fibrosis by immuno histochemistry.
- To study the expression of loricrin in formalin fixed paraffin embedded tissue specimens of hyperkeratosis by immuno histochemistry.
- To study the expression of loricrin in formalin fixed paraffin embedded tissue specimens of normal mucosa by immuno histochemistry.
- To compare the expression of loricrin in formalin fixed paraffin embedded tissue specimens of Oral submucous fibrosis, hyperkeratosis and normal mucosa by immuno histochemistry.

HYPOTHESIS: (NULL)

There is no difference in the expression of Loricrin in the epithelium of oral submucous fibrosis, hyperkeratosis and normal mucosa.

HYPOTHESIS (ALTERNATE)

There is a difference in the expression of Loricrin in the epithelium of oral submucous fibrosis, hyperkeratosis and normal mucosa.

Materials and Methods

STUDY SETTING

The study was conducted in the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, using formalin fixed paraffin embedded tissues.

It was a retrospective study, done to evaluate the expression of loricrin in oral submucous fibrosis, hyperkeratosis and normal mucosa using immunohistochemistry in archival formalin fixed, paraffin embedded tissue specimens. The study was approved by the Institutional Review Board. **(Annexure I)** and a protocol was submitted for the same. **(Annexure II)** Informed consent was obtained from patients for samples of normal buccal mucosa for the control group.

STUDY SAMPLE SIZE

The study material comprised of 73 formalin fixed, paraffin embedded tissue specimens (archival blocks).

Sample size calculation-The above sample size was calculated for a power of 80% at 5% significance level.

STUDY SUBJECTS (n=73)

The study comprised of 3 groups

Group I (n=11)

Eleven archival blocks of clinically normal buccal mucosa, obtained from patients reporting to the outpatient department of oral and maxillofacial surgery for elective removal of impacted third molar constituted group I.

Group II (n=32)

Thirty two archival blocks of histopathologically confirmed hyperkeratosis tissue specimens. (Both frictional keratosis and epithelial dysplastic lesions with hyperkeratosis were included within this group)

Group III (n=30)

Thirty archival blocks of histopathologically confirmed oral submucous fibrosis tissue specimens.

METHODOLOGY

1. Tissue samples of oral submucous fibrosis (n=30),hyperkeratosis (n=32) and normal mucosa (n=11) were drawn from the archives of department of oral and maxillofacial pathology.
2. Four micron thick sections were cut and used for routine Haematoxylin and Eosin (H & E) staining
3. H & E staining and immunohistochemical (IHC) staining was performed.
4. This project was approved by The Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai and patient consent was taken.

All materials were procured from MERCK^{TM} and were of analytical grade unless mentioned.

HAEMATOXYLIN & EOSIN STAINING

REAGENTS

- Harris's haematoxylin
- 1% acid alcohol
- Eosin

PROCEDURE

- The slides were dewaxed in xylene and hydrated through graded alcohol to water.

- The sections on the slides were flooded with Harris's haematoxylin for 5 minutes.
- The slides were washed in running tap water for 5 minutes.
- The slides were differentiated in 1% acid alcohol for 5 minutes.
- The slides were washed well in running tap water for 5 minutes.
- The tissue sections on the slides were then stained in eosin for 30 seconds.
- The slides were washed in running tap water for 1 minute.
- The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope (LM).

IMMUNOHISTOCHEMISTRY (IHC)

ARMAMENTARIUM

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Couplin jars
- Measuring jar
- Weighing machine
- APES coated slides
- Slide carrier
- Aluminum foil
- Micro-pipettes
- Toothed forceps

- Electronic timer
- Beakers
- Rectangular steel tray with glass rods
- Sterile gauze
- Cover-slips
- Light microscope

REAGENTS USED

1) Concentrated Hydrochloric acid, Analytical grade
2) Laxbro solution.
3) APES (3 amino propyl tri ethoxy silane)
4) Acetone
5) Citrate buffer
6) Phosphate Buffer Saline (PBS)
7) 3% Hydrogen peroxide (H_2O_2)
8) Deionised distilled water
9) Absolute alcohol
10) Xylene

ANTIBODIES USED

1. Primary antibody – Loricrin polyclonal antibody, Abcam, Allied scientific was used in a concentrated form.

2. Secondary antibody –Expose Mouse and Rabbit specific HRP/DAB detection IHC kit containing Poly Horse Radish Peroxidase –pre titrated anti-species

immunoglobulin labeled with enzyme polymer (Goat anti rabbit HRP conjugate) and mouse specifying reagent, and liquid DAB- Diamino-benzidine-chromogen .

IHC PROCEDURE

PRETREATMENT OF THE SLIDES

- The slides were first washed in tap water for few minutes
- The slides were then soaked in detergent solution for 1 hour
- After 1 hour, each slide was brushed individually using the detergent solution and were transferred to distilled water.
- The slides were washed in two changes of distilled water
- The slides were washed in autoclaved distilled water
- The slides were immersed in 1 N hydrochloric acid (HCL) (100 ml HCl in 900 ml distilled water) overnight
- The following day, slides were taken out of acid and washed in two changes of autoclaved distilled water
- All the slides were then transferred to slide trays, wrapped in aluminium foil and baked in hot air oven for 4 hours at 180 degrees centigrade.

APES (3 Amino propyl tri ethoxysilane) coating

Slides first dipped in couplin jar containing acetone for 2 minutes

Dipped in APES for 5 minutes

Dipped in two changes of distilled water for 2 minutes each

Slides left to dry

PREPARATION OF PARAFFIN SECTIONS

After the slides were dry, tissue section of 5 micron thickness were made in a rotary microtome. The ribbons of tissue section were transferred onto the APES coated slide from the tissue float bath such that two tissue bits come on to the slide with a gap in between. One of the tissue sections was labelled positive (P) and the other negative (N).

PROCEDURE

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. The slides were then transferred to citrate buffer with a pH of 6.0 and autoclaved for antigen retrieval at 15 lbs pressure for 30 minutes. The slides were dipped in 3 changes of phosphate buffered saline for 5 minutes each. Circles were drawn around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. Slides were then treated with 3% hydrogen peroxide for 15 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non-specific staining. The tissues were incubated in protein blocking

serum for 10 minutes in an enclosed hydrated container. Then the slides were wiped carefully without touching the tissue section to remove excess of blocking serum. The primary antibody, Loricrin polyclonal antibody was added to P tissue on the slide while PBS was added to the N tissue on the slide. The slides were incubated for one hour. Then the slides were wiped carefully without touching the tissue section to remove excess of antibody and washed with three changes of cold PBS for 5 minutes. A drop of Mouse specifying reagent was added to both sections on the slide and allowed to stand for 15 minutes and was followed by PBS buffer rinse two times. Then a drop of Goat anti rabbit specific HRP conjugate is added to both sections and allowed to stand for 10 minutes. (Expose Mouse & Rabbit specific HRP/DAB detection kit:Ab80436-15ml)

Later slides were rinsed 4 times in PBS. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of DAB was added to the sections. Slides were then rinsed in PBS to remove excess chromogen and counter stained with hematoxylin for 30 seconds . The tissue sections were mounted with DPX. The slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

Positive Control: A foreskin specimen procured after circumcision for Phymosis was fixed, processed, embedded, sectioned and stained in the same manner and used as positive control. One positive control tissue slide was included for each batch of staining.

IHC PROCEDURE FLOW CHART

APES coated slides with 2 paraffin embedded tissues

Placed in xylene twice (5 minutes each)

↓

Placed in 100% isopropanol (5 minutes)

↓

Placed in 70% isopropanol (5 minutes)

↓

Washed in distilled water (5 minutes)

↓

Kept in citrate buffer at pH 6 and autoclaved for antigen retrieval and bench cooled for 40 minutes

↓

Washed in PBS thrice (5 minutes)

↓

Placed in 3% hydrogen peroxide (15 minutes)

↓

Protein blocking serum added and incubated for 10 minutes

↓

Primary antibody added to the specimen and incubated for one hour

↓

Washed in PBS thrice (5 minutes each)

↓

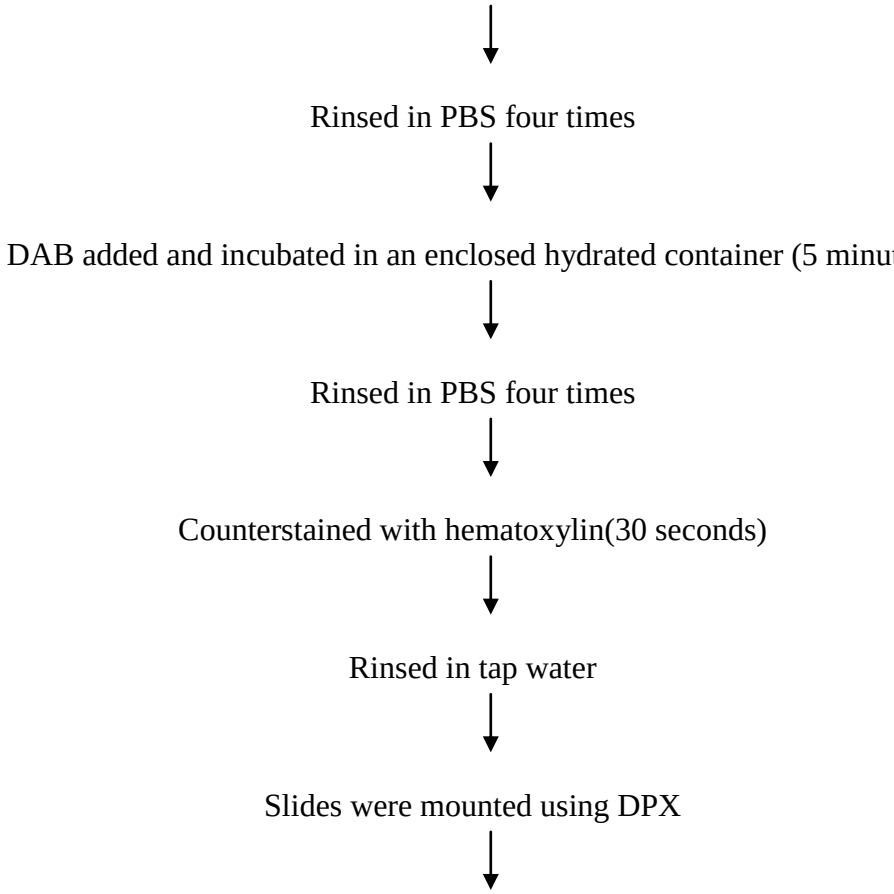
Secondary antibody added and incubated in an enclosed hydrated container (15 minutes)

↓

Rinsed in PBS twice

↓

HRP -Goat anti rabbit specifying reagent added and incubated (15 minutes)



Criteria for evaluation of Loricrin staining

The parameters used to evaluate Loricrin (LOR) staining were as follows

1. **Tissue localization of stain** –Loricrin staining is localized to the epithelium.

   Seen in the stratum granulosum and stratum corneum of stratified squamous epithelium in keratinized epithelia, while it is absent in non keratinising epithelium of the oral mucosa.

2. **Cellular localization of stain** –The Stain is localized to both the cytoplasm and Nucleoplasm of the cells.

3. **Degree of positivity** –Each case was graded as (-) nil or absence of stain, (+) mild, (++) moderate and (+++) intensively stained based on the intensity of staining taken up by the tissue as observed by three blinded observers independently.
4. **Statistical analysis-**Data were entered and analyzed using SPSS ™ software (version 17.0). Pearson's Chi-square test was done to compare intensity of staining between the groups and a P value <0.05 was considered statistically significant. Kappa analysis was done to compare the intensity of loricrin staining as observed between three observers.

Review of Literature

Epithelial tissues are the main appendages that protect the body's internal tissues from the environmental stresses, chemical damage and bacterial infection. The stratified epithelia of the skin and oral mucosa are examples of the toughest and most protective epithelia.[8] Keratinizing epithelia such as the epidermis and oral gingiva that have to withstand severe physical and chemical forces produce a toughened structure, the cornified cell envelope. The stresses or insults have been implicated as a causative factor in the progression from a potentially malignant disorder to a condition of full blown cancer.[9]

The coarse fibres of areca nut and the continuous friction from occluding teeth are the major causes of mechanical stress to the oral mucosa in oral submucous fibrosis and frictional keratosis (hyperkeratosis) respectively. Hence, the role of Loricrin in oral epithelium needs to be studied in these two lesions which when identified, serves as a early diagnostic marker in identifying the potentially malignant disorders, thereby leading to a better prognosis and better treatment option for the patient at risk from oral cancer.

Oral submucous fibrosis (OSF)

Submucous fibrosis may be defined as an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it, is always associated with juxtaepithelial inflammatory reaction followed by a fibro elastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat.[10]

In 1952, Schwartz described five Indian women from Kenya with a condition of the oral mucosa including the palate and pillars of the fauces, which he called "Atrophia idiopathica (tropica) mucosae oris". Later it was termed oral submucous fibrosis(OSF) by Joshi in 1953.[11]

The other names for this condition are "diffuse oral submucous fibrosis", "idiopathic scleroderma of the mouth", "idiopathic palatal fibrosis", "sclerosing Stomatitis" and "juxtaepithelial fibrosis"[10]

Geographical distribution and Epidemiology

Worldwide estimates in 1996 indicate that 2.5 million people were affected by this disease. In 2002, the statistics for OSF from the Indian continent alone was about 5 million people (0.5% of the population of India)

Cases have also been reported in other parts of the world among Indian settlers or people of ethnic origin. Potential for malignant transformation into squamous cell carcinoma has been observed in one third of the cases with submucous fibrosis in a clinical study with 650 patients in India.[12] Another study on oral cancer and precancerous lesions in a rural Indian population revealed the malignant transformation rate of OSF to be 7.6% (5 of 66) over a 17-year period (median observation, 10 years)[13]

Women seemed to show a higher prevalence that seems to be related to the deficiency of iron and vitamin B complex seen among many Indian women[12]

The prevalence of oral submucous fibrosis, leukoplakia, and oral lichen planus were 0.55%, 0.59% and 0.15% respectively in a cross sectional study conducted among 2017 consecutive patients from sub urban and rural areas of Chennai

Tamilnadu during a period of three months with their prevalence directly proportional to their habits of chewing and smoking.[12] A case control study in Taiwan with a study group of 35 cases and 100 controls showed a high correlation between this condition and areca nut chewing. [14] According to J.J .Pindborg, the greater prevalence in the Indian sub continent is related to its ethnical use of areca nut products. A wide range in the age of patient are seen with the majority in the 20-40 age group. with a higher prevalence among the south Indians than north Indians.[10]

Aetiology of OSF:

The cause of the OSF, is still obscure with the various hypotheses put forward, pointing to a multifactorial origin. Hypersensitivity to chilli and betel nuts and nutritional deficiencies have all been suggested at various times to account for the pathogenesis of the disease, with emphasis on locally acting irritants on the oral mucosa. [15]

Capsaicin of chillies has been associated in the pathogenesis of OSF in some studies while no evidence for the same has been proposed by Hamner *et al* in experiments with hamster cheek pouch.[16,17] Short term experimental tests on experimental animals with chilli extracts has been found to be mutagenic, enhancing the tumorigenicity of tobacco products. Increased chilli consumption has also been shown to increase the risk of cancers in the upper digestive tract in a dose dependent manner [10] However no association between chilli and OSF was found in a Taiwanese study of 35 cases with 100 controls.[14]

OSF is frequently associated with nutritional deficiency which is usually secondary to the condition.[17]An increased mucosal susceptibility to irritants like

areca nut and chillies are seen in deficiencies of vitamin and iron which leads to derangement in the inflammatory reparative response in the lamina propria with resultant defective healing and scarifications[18]. Genetic susceptibility for this condition is implicated to the action of extrageneous factors like tannins and areca alkaloids and in conditions where there are no habits of areca nut chewing. An autoimmune explanation could also be given to OSF due to its similar appearance to other disorders like scleroderma in both its clinical presentation and its histologic characteristics.[19] Canniff *et al* has reported an increase in the DR3 antigens in OSF along with serum immunoglobulin and auto antibodies similar to an increase in the DR gene seen in scleroderma.[20,21]

Areca nut chewing plays a major role in the aetiology of OSF, and has been said to play a role in the pathogenesis, by stimulation of the fibroblast by its constituents notably the alkaloids resulting in phenotype alteration and increased collagenesis, which could be seen in tissue culture studies involving cell lines from human fibroblasts which have reproduced connective tissue disorders as they maintain their phenotype in culture and their properties *in vivo*. Fibroblasts metabolize the alkaloid arecoline to arecaidine and its esters which in turn acts as a stimulator of the proliferation of fibroblasts and collagen synthesis. The relative risk of OSF, increased with the duration as well as the frequency of the areca nut (main ingredient in Mawa) chewing habit.[15]

The severity and the time taken for the development of the disease may also vary according to the preparation of areca nut consumed. The commercially freeze dried products such as pan masala, gutka and mawa (areca and lime) have high concentrates of areca nut per chew and appear to cause OSF more rapidly than by

self prepared conventional betel quid which contain smaller amounts of areca nut.[22]

Clinical presentation in oral submucous fibrosis.

Oral submucous fibrosis has an insidious onset of about 2 to 5 years presenting with prodromal symptoms which include ,appearance of blisters especially in the palate, burning sensation on consuming spicy foods, ulcerations, recurrent generalized inflammation of the oral mucosa, defective gustatory sensation and dryness of the mouth, with periods of waning and exacerbations varying from 3 months to 1 year.[4]

Focal vascular manifestations in early OSF present clinically as petechiae, in the absence of any other blood dyscrasias might be a hypersensitive reaction of the oral mucosa in response to an external stimulant like areca nut and chillies revealing a slightly atrophic epithelium with numerous dilated and blood filled capillaries seen juxtaepithelially.[20]

The major presenting complaint is a progressive inability to open the mouth because of the accumulation of inelastic fibrous tissue in the juxtaepithelial region of the oral mucosa, along with concomitant muscle degeneration A study which enabled assessment of alterations in oral submucous fibrosis by establishing the parameters of normal mouth opening (mo), tongue protrusion (tp) and cheek flexibility (cf) was done to determine early changes was done by Ranganathan et al[23]

Histological Features in oral submucous fibrosis.

Histologically, OSF is characterized by juxtaepithelial fibrosis, along with atrophy or hyperplasia of the overlying epithelium, keratinizing metaplasia, and accumulation of hyalinised collagen beneath the basement membrane with a progressive loss of vascularity. Often, a variable infiltration of the lamina propria with chronic inflammatory cells also is evident. (Utsunomiya *et al)*[24]

Changes observed in the epithelium of oral submucous fibrosis.

OSF shows ultra structural changes at both the light microscopic and electron microscopic level. Variations can be seen depending on the site of the lesion and the severity of the condition observed clinically.OSF is a connective tissue disorder and the changes seen in the epithelium are only secondary to the connective tissue changes. The epithelium is dependent on the connective tissue for its nutrients and shows a varied presentation with a normal appearance to a atrophied or a hyper plastic one. [20]

Hyper plasticity could be due to an protective response to the local irritants Atrophic epithelium with loss of reteridges is seen in a majority of the cases when compared with normal mucosa. Intracellular oedema, signet cells with focal areas of dysplasia is seen frequently. Keratinisation tends to be higher in atrophic or hyper plastic epithelium along with vacuolization of the prickle cell layer and increased mitosis. An increase in the intercellular spaces leads to a greater permeability to water and other serum derived antibodies as well as to local irritants.

The haematoxylin and eosin stained sections are helpful in characterizing the changes that are observable within the connective tissue and have four consecutive stages namely the presence or the absence of oedema, state of the blood vessels Nature of the collagen bundles, the overall fibroblastic responses along with the predominant inflammatory cell infiltrate.[20]

EM changes in the epithelium of oral submucous fibrosis:

In the epithelium the small red pin sized areas seen with the naked eye are suggested to be areas with complete loss of epithelium focally, while ultra structurally there is reduction in the number of cell layers in association with intracellular oedema. This oedema in turn causes loosened inter digitations and widened intercellular space with the cell organelles with reduced or absent tonofilaments .The cells therefore appear darker with shrunken cytoplasm pyknotic nuclei and marginated chromatin.[24,25]

SEM changes in the epithelium :

Scanning electron microscopy(SEM) reveals obliteration of the elevated ridges of the cell boundaries without alteration seen in the micro rugae. Some areas showed desquamation of the squamous epithelial cells along with complete focal loss of the covering epithelium ,resulting in exposure of the sub epithelial connective tissue. The SEM also showed the presence of microbes on the epithelial cell surface which was an significant find as the invasion of the microbes subepithelially would lead to an altered oral homeostasis with resultant proliferation of the microbes enhancing the importance of the disruption of the oral barrier.[26]

Haque *et al* in a study investigated the presence and distribution of inflammatory cells and MHC class II antigen expression by epithelial and immunocompetent cells using a three-stage immunoperoxidase method on frozen sections of thirty OSF sections. The cell population detected in OSF showed higher numbers of CD3 and HLA-DR-positive cells in the basal layer of the epithelium, juxtaepithelium and in the lamina propria in a similar distribution to that of CD3 cells compared with the normal tissues. The increased evidence of CD4 and HLA-DR-positive cells in OSF tissues suggests that most lymphocytes were activated and shows an increased presence of Langerhans cells. The presence of these immunocompetent cells and high ratio of CD4 to CD8 in OSF tissues suggest an ongoing cellular immune response leading to a possible imbalance of immunoregulation and alteration in local tissue architecture.[27]

The electron microscopic study in 11 OSF cases revealed densely packed collagen bundles in the lamina propria seen close to the epithelial connective tissue junction with thinner type III collagen fibrils present here along with a variation of type I fibrils which form the bulk of the fibrils. Inspite of their increased density ,they were found to be morphologically normal. This is also confirmed by polarization and special staining with Sirius red.[20]

Treatment of OSF:

Though many treatment protocols for oral submucous fibrosis have been proposed, there are few if any controlled studies evaluating the effectiveness of treatment protocols Treatment strategies that have been tried, are topical &

systemic steroids supplements of vitamins, minerals & micronutrients, use of enzymes, repeated dilatations with physical devices & surgery. Local & systemic application of glucocorticoids, placental extracts & immunomodulators are most commonly used as they suppress the inflammatory reaction, decrease collagen formation. In severe cases, surgical intervention is the only treatment modality, but relapse is a major problem.[28] Inspite of the various treatment options available the best way to stop progression is cessation of habits and identification of the disease condition by its association with the habit of areca nut and betel quid chewing, Immunohistochemical evaluation at an early stage acts as an valuable adjuvant and helps in identification of the stage of the disease and helps us to provide treatment which would provide a better treatment prognosis.

Association of areca nut and OSF and micro trauma

The IARC Monographs (2004) found that there is a relationship between the use of areca nut extract and development of OSF, prompting the World Health Organization to classify areca nut as a group I carcinogen.

Areca nut is consumed in various forms with and without tobacco such as betel quid (areca nut + slaked lime + betel leaf), pan masala (powdered areca nut with additives + flavouring agents + tobacco) and raw areca nut (seeval flakes and kotta paaku granules). In India, there are regional variations in the type of areca nut product used [22]

Paymaster in a clinical study of 650 cases in Indian patients reported malnutrition with subsequent avitaminosis and long-continued chewing of betel nut with tobacco as predisposing factors to oral cancer.[11]

A case control study done by Ranganathan et al in south India with a study group of 185 patients with a male to female ratio of OSF cases was 9.9:10. Indicate that OSF is significantly associated with use of pan masala and pan masala plus areca nut, with or without concurrent alcohol use, with the risk being greatest for pan masala (processed areca nut without betel leaf; odds ratio, 81.5). Followed by areca nut plus alcohol use (odds ratio, 69.9). Tobacco smoking and use of alcohol without use of areca nut products is not associated with OSF and are not risk factors for OSF alone or in combination.[29]OSF is a clinically significant disorder characterized by fibrosis of the sub mucosal tissues of the mouth, which limits movement of the oral mucosae and tongue, giving rise to microstomia and eventual dysarthria and dysphasia, with a significant risk of potentially-malignant and malignant disease predominantly caused by both long-term use of areca nut-containing agents and a genetic basis.[29]

A study by Chang *et al* showed that areca nut and Arecoline in betel quid may inhibit the growth of oral mucosal fibroblasts (OMF) and keratinocytes showed that areca nut extract (100-800 microgram/ml) and arecoline (20-120 micro mol) inhibited the growth of oral KB cells (a subtype of cultured cancer cell line) by 36-90% and 15-75%, respectively, indicating that areca nut ingredients are crucial in the pathogenesis of OSF and oral cancer by differentially inducing the dysregulation of cell cycle control, Glutathione (GSH) level and intracellular hydrogen peroxide (H_2O_2) production, these events being not coupled with cellular changes.[30]

Both areca nut and betel quid act as irritants to the oral mucosa, mechanically and chemically. Arecoline, the most abundant alkaloid undergoes

nitrosation giving rise to N-nitrosamines, which has a cytotoxic effect on cells, at the same time promoting synthesis of collagen.

The constant contact between the mixture and oral mucosa which occurs due to the habit of placing the betel quid in the buccal vestibule for about 15 min to an hour with the action being repeated five to six times a day, depending upon the individual's chewing habit, leads to absorption and metabolization of the alkaloids and flavonoids in the betel quid which acts betel quid constituents and their metabolites, the coarse fibres of areca nut also cause mechanical irritation to the oral mucosa.[15]

The micro trauma produced by the friction of coarse fibres of areca nut also facilitates the diffusion of betel quid alkaloids and flavonoids into the sub-epithelial connective tissue, resulting in juxtaepithelial inflammatory cell infiltration, which turns into a chronic inflammatory cell infiltrate due to the persistent habit. Atrophy and ulceration of the mucosa follow the initial irritation. Inflammation is characterized by the presence of activated T cells, macrophages, with elaboration of various chemical mediators of inflammation like prostaglandins (PGs) whose secretion by oral keratinocytes in response to areca nut extract has been shown .Aberrant and persistent tissue inflammation are crucial for the occurrence of cancer and tissue fibrosis. Thus, it can be considered that induction of oral mucosal inflammation by betel quid ingredients to be a critical event in the pathogenesis of OSF. Cytokines like interleukin 6, Tumour necrosis factor (TNF), interferon alpha and growth factors like TGF-b and FGF are synthesized at the site of inflammation.[15]

Fibroblast growth factor (FGF) also act as mitogens for endothelial cells and other fibroblast like cells and are involved in angiogenesis, directing endothelial cell migration, proliferation and plasminogen activator synthesis. Both activated macrophages and damaged endothelial cells produce FGF2 which is also a mitogen for keratinocytes indicating a role in skin wound healing with *in vivo* experiments showing an acceleration in granulation tissue formation fibroblast proliferation and collagen accumulation, with enhancement of vascularisation and re-epithelialisation.[15]

Fibrosis of a reparative type usually develops after an initial insult which leads to cellular injury, as might be the case in OSF following mechanical insult by the coarse fibres of areca nut or pulmonary fibrosis following asbestos exposure, with its pathophysiology following a parallel path with wound healing and tissue repair. Excessive matrix deposition not triggered by tissue injury but by a direct stimulation from local factors also results in fibrosis, like interstitial and perivascular fibrosis in heart compromising function.

Therefore, the fibrotic response appears to be determined by three factors:

(1) A continuous insult or stimulus, suggesting that the fibrotic process is an on-going event;

(2) Excessive synthesis of collagen and other extra cellular matrix (ECM) components; and

(3) Decrease in the resolution owing to down regulation of the degradative enzymes involved in removing the scar tissue. At present, it is not clear if factors such as genetic predisposition are important in determining the susceptibility to developing these conditions[31]

Increased susceptibility among individuals who are anaemic due to iron or vitamin B12 deficiencies has been demonstrated. This could be due to increased fragility of the mucosa by which there is more betel quid absorption.

TGF-β1 is a key regulator of ECM assembly and remodelling. The action of TGF-β on the genes implicated in the formation and degradation of the ECM is mostly exerted at the transcriptional level through ill defined intracellular pathways. The molecular events usually follow the collagen production pathway and collagen degradation pathway, as regulated by TGF-β and the flavonoids present in areca nut. The risk for oral leukoplakia also increased with the level of intensity, suggesting a dose–response relationship between areca-nut chewing and oral leukoplakia[15].

Cornified cell envelope (CE)

The Cornified cell envelope CE is a 15nm thick layer of insoluble protein,[34] which is seen as an electron dense band on the inner side of the plasma membrane of keratinocytes during terminal differentiation. It first appears in the most superficial granular or transitional cells of terminally differentiating stratified squamous epithelia and is composed of a 10-nm-thick layer of a cross-linked sheath of proteins and a 5-nm-thick layer of ceramide lipids that is covalently attached to the proteins. Various proteins, which are mostly transglutaminase (TGase) substrates, are included in the CE. [32,33]

Factors influencing CE formation

Diverse environmental stimuli like contact allergens and ultraviolet light trigger a cutaneous inflammatory response by directly inducing epidermal keratinocytes to elaborate specific pro-inflammatory cytokines and adhesion molecules leading to activation of dermal microvasculature endothelial cells and selective accumulation of specific mononuclear cells in the dermis and epidermis, indicating that keratinocytes may act as a "signal transducer" capable of converting exogenous stimuli into the production of cytokines, adhesion molecules, and chemo tactic factors (acting in an autocrine and paracrine fashion) responsible for initiation of "antigen-independent" cutaneous inflammation.[34]

This initiation phase facilitates production of tumour necrosis factor–alpha and interferon gamma by the antigen dependent pathway keratinocyte/T cell/antigen-presenting dendritic cellular associations. The direct activation of keratinocytes, with their ability to produce the complete repertoire of pro-inflammatory cytokines, can profoundly influence endogenous and recruited immunocompetent cells, thereby providing the critical trigger responsible for the swift and clinically dramatic alterations that occur following contact between the epidermis and a host of "noxious" agents.[34]

Skin functions as a stable, physical and chemical barrier from environmental influences. The CE is a critical structure for barrier function at the outermost layer of the skin epidermis. For the formation of CE in terminal keratinocyte differentiation, covalent cross-linking of constituting proteins such as involucrin, loricrin, small proline-rich protein is essential and requires the calcium

dependent enzyme transglutaminase (TGase) for catalyzing the isodipeptide bond formation.

Among proteins TGases 1,3 and 5 participate in CE formation . TGases 1 and 3 are activated by limited proteolysis during differentiation of the keratinocytes and this contributes to the sequential cross-linking of substrates, utilizing different glutamine and lysine residues, indicating that both enzymes have distinctly complementary and essential functions in the utilization of loricrin for CE assembly *in vivo* [35] therefore disorders of TGases leads to irregular phenotype in the skin.[33] Loricrin contains three glycine rich domains which are thought to form uniquely flexible glycine loops, interspersed by glutamine-rich motifs and flanked by lysine- and glutamine-rich amino and carboxy terminal domains [36,37]

An evolutionary analysis of Keratinisation in association with CE formation.

Alibardi L *et al.*[38]in a study analyzed the comparative aspects of epidermal keratinisation in vertebrates, with emphasis on the evolution of the stratum corneum in land vertebrates .The Fish epidermis does not contain proteins connected with interkeratin matrix and corneous cell envelope formation, Loose keratin filaments are glued together by Mucus-like material . In the case of amphibians a cell corneous envelope forms, but matrix proteins, aside from mucus/glycoprotein, are scarce or absent while specific proteins associated with keratin become relevant for the production of a resistant corneous layer in reptiles, birds, and mammals. In reptiles some matrix, histidine-rich and sulfur-rich corneous cell envelope proteins are produced in the soft epidermis. In avian soft

epidermis low levels of matrix and cornified proteins are present while lipids become abundant.

In mammalian keratinocytes, inter-keratin proteins, cornified cell envelope proteins, and transglutaminase are present. Topographically localized areas of dermal-epidermal interactions in amniote skin determine the formation of skin derivatives such as scales, feathers, and hairs. New types of keratin and associated proteins are produced in these derivatives. In reptiles and birds, beta-keratins form the hard corneous material of scales, claws, beaks, and feathers. Small sulfur-rich and glycine-tyrosine-rich proteins form the corneous material of hairs, horns, hooves, and claws in mammals. Molecular studies on reptilian beta-keratins show they are glycine-rich proteins. They have C- and N-terminal amino acid regions homologous to those of mammalian proteins and a central core with homology to avian scale/feather keratins.[8,38] These findings suggest that ancient reptiles already possessed some common genes that later diversified to produce some keratin-associated protein in extant reptiles and birds, and others in mammals. The evolution of these small proteins represents the more recent variation of the process of cornification in vertebrates [38]

Loricrin

Loricrin is a insoluble polypeptide with a conserved epitope and is a late differentiation marker protein that is introduced into the scaffold of the cornified envelope by virtue of its cross linking and binding property and enhances the property of protective barrier of the corneocyte**.**

Loricrin as a major component of CE.

The process of terminal differentiation in stratified squamous epithelia such as the epidermis, leads to a 15-nm thick layer of protein being deposited on the intra-cellular surface of the cell periphery. Steinert PM and Marekov LN in their study show that this cornified cell envelope (CE) plays a critical role in barrier function of the tissue and for the organism and is made up of a complex amalgam of proteins cross-linked by isodipeptide bonds formed by the action of transglutaminases, like involucrin, cystatin α, several Small proline-rich proteins (SPR1, SPR2, in epidermis as well as SPR3 in cultured keratinocytes) loricrin and possibly trichohyalin, Filaggrin, Keratin intermediate filaments (KIF)), and a putative cysteine-rich protein that may be elafin and is regulated by a rise in intra cellular calcium.[39]

The presence of loricrin as a structural protein and a major component of the cornified epithelium was illustrated by using the indirect mathematical modelling method, and has been estimated that loricrin is the major component 70%, followed by filaggrin 8%, elafin 6%, small proline rich proteins (SPRs) and cystatin α 5% each, and involucrin and keratin intermediate filaments about 2% each. This large amount of loricrin is consistent with the abundance of its mRNA in the epidermis and has been done by Western blotting or other biochemical techniques. Transgenic experiments suggested that loricrin is one of the last components added during CE assembly by addition to a pre-existing scaffold of proteins such as involucrin and cystatin α.Proteins pancornulins and involucrin

were found to be components released on treating isolated CE's of cultured epidermal keratinocytes with chemical solvents for protein characterization.

Alternatively after the digestion of isolated CE's from human foreskin epidermis to their constituent amino acids with low specificity proteases the remaining insoluble CE remnants were subjected to the mathematical modelling to estimate the presence of proteins present, after initial digestion with the trypsin and proteinase K digestion. This was followed by immunogold electron microscopy of the remnants using a series of monospecific antibodies which removed primarily keratin and Filaggrin epitopes from one side of the CEs. During 24 h of digestion with proteinase K, only loricrin, SPR, and the novel protein elafin were removed primarily, based on both amino acid composition and immunogold criteria.[40]

From the following experiments it was seen that the outer (cytoplasmic surface) third of the CE consists mostly of loricrin/SPRs/Filaggrin; the middle third consists of elafin/loricrin/SPRs; and the innermost third adjacent or attached to the lipid envelope consists of involucrin and cystatin α and perhaps other as yet unknown proteins. Also the keratin intermediate filaments (KIF) may be buried throughout the CE However, the most rigorous evidence for the involvement of a protein in CE structure, that circumvents the concerns of degraded, loosely-associated, or contaminating solubilised proteins, would be to demonstrate identifiable protein sequences directly adjoined by isodipeptide cross-links in isolated CEs, as has been done in preliminary experiments for Loricrin, proving that. Peptides from the outer (cytoplasmic) third of CE structure are mostly Loricrin and SPRs.[40]

In vivo, loricrin is expressed in all mammalian stratified epithelia tested so far. However, the highest levels of expression are found in humid tissues such as newborn epidermis, the epithelia of oral and anal mucosa, oesophagus, foreskin, vagina and the epidermal parts of sweat ducts. [41,42]

Structure of loricrin

According to Yoneda *et al* [43] Loricrin is the major protein component of the cornified cell envelope of terminally differentiated mammalian epidermal (stratum corneum) cells. Isolation and characterization of the human loricrin gene by using a specific human cDNA clone was done. Loricrin was found to have a very simple structure of a single intron of 1188 base pairs (bp) in the 5'-untranslated region with no introns in coding sequences. Loricrin a single copy gene was localized to the chromosome 1q21[41,8] by using rodent-human somatic cell hybrids, followed by in situ hybridization with a biotin-labelled genomic DNA clone.[42]

Genomic DNA analysis using PCR (Polymerase chain reaction) from different individuals showed that human loricrin consists of two allelic size variants, which is due to sequence variations in its second glycine loop domain, and these variants segregate in the human population by normal Mendelian mechanisms. Multiple sequence variants are seen within these two size class alleles due to various deletions of 12 basepairs (4 amino acids) in the major loop of this glycine loop domain. By use of a specific loricrin antibody, and immunogold electron microscopy it is seen that loricrin initially appears in the granular layer of human epidermis and forms composite keratohyalin granules with profilaggrin, but

localizes to the cell periphery (cell envelope) of fully differentiated stratum corneum cells.[43]

Schmuth M *et al* ,[44] in their study showed that Loricrin which is a glycine–serine–cystine-rich structural protein synthesized in the stratum granulosum (SG) layer, migrates to the cell periphery, the outermost layer of the SG. It is then deposited beneath the plasma membrane, cross-linked to several other cytosolic proteins like involucrin, small proline rich proteins (SPRR), elafin, repetin, S100, and up to 20 other proteins by means of transglutaminase-1, forming the cornified envelope (CE) contributing to its mechanical resistance.

The CE not only protects against external injury, but also serves as scaffold for the deposition of the extracellular lamellar bilayers which are in turn linked to the CE through an interposed layer of covalently bound -hydroxyceramides, the cornified-bound lipid envelope (CLE).[44]

The extracellular lamellar membranes comprised mainly of cholesterol, free fatty acids, and ceramides seal the extracellular spaces against excess, bidirectional movement of water, electrolytes, and xenobiotes. The extracellular lamellae is derived from the secretion of lamellar body (LB) contents at the SG–stratum corneum (SC) interface by a process that occurs immediately prior to loricrin cross-linking into the CE . The combination of a hydrophobic extracellular compartment, linked to a mechanically resistant protein scaffold, not only provides an effective interface between the body and the environment but it also mediates additional, protective cutaneous functions, such as antimicrobial, antioxidant, chemical, and mechanical defence.[32]

In the initial stage, involucrin an CE constituent along with the desmosomal proteins such as envoplakin and periplakin forms a "scaffold" by cross-linking to produce a monomolecular layer beneath the plasma membrane. Subsequently, cross-linking of involucrin occurs with ceramide which replaces the lipid bilayer at the plasma membrane leading to the fixation of the scaffold proteins at the cell periphery. This scaffold serves as a platform for the subsequent addition of various reinforcement proteins, including loricrin and SPRs. These structural proteins, before translocation to the cell periphery, are cross-linked to form homo- and hetero dimers. In the final dead cornified cells, the completed structure is stabilized by a covalent attachment of keratin intermediate filaments to the CE. Among the proteins that participated in CE formation, the major substrate molecules are involucrin, loricrin and SPR. These proteins have structural properties of glutamine- and lysine-rich residues that are commonly modified by transglutaminases . (CE- Precursor proteins)[32,33]

Name	Gene locus	Size (kDa)	Human foreskin site	Identified in vivo
Involucrin	1q21	(EDC)65	2-5%	Yes
Loricrin	1q21	(EDC) 26	80%	Yes
SPRs	1q21	(EDC) 6-26	3-5%	Yes

(EDC- Epidermal differentiation protein.) [32]

Loricrin, is the most abundant protein comprising about 75% - 80% of the total CE protein mass, and is insoluble mainly due to its high glycine content. Loricrin has the highest glycine content of any known protein in biology [37,43]It is initially sequestered into loricrin granules with an unique amino acid sequence rich in glycine, serine, and cysteine residues. Glutamine- or glutamine/lysine residues are also frequently observed. Owing to its high rate of expression and low solubility, loricrin forms spherical inclusions, called L-bodies in newborn mouse skin, human foreskin and acrosyringium, but it is diffusely distributed in the cytoplasm of adult epithelia.

Functions of loricrin

In vitro cross-linking experiments using recombinant human loricrin have demonstrated that the cross-linked loricrin functions as a major reinforcement protein for the CE on the cytoplasmic face of the structure- or internal 'wet' epithelia.

While a greater content of loricrin is seen in the epidermal CEs, it is rather less (30-50%) in the CEs of certain internal epithelia such as Oesophagus, palate, buccal mucosa and is not expressed in many other internal epithelia . Phorbol esters, cell confluence, and Ca2+ induce expression of loricrin presumably through signals acting through an AP1 site and it occurs late during the terminal differentiation program of these tissues.[32,36,45,46]

Expression of loricrin in the epidermis is also regulated with the presence of certain immunoglobulin-like cell –cell adhesion molecule like Nectin that plays essential roles in the initial step of formation of adherens junctions and tight

junctions. Newborn nectin-1-/- pups showed shiny and slightly reddish skin with marked reduction in the expression of loricrin, with compensatory elevation in the amounts of repetin and small proline rich proteins, to overcome the impaired expression of loricrin. However, cornified cells from nectin-1-/- mice were sensitive to mechanical stress. Moreover, Ca2+-induced activation of ERK through Rap1 and expression of loricrin were reduced in primary cultured nectin-1-/- keratinocytes.[5]

Loricrin and OSMF

In a study by Li N *et al*, microarray analysis was done and the mRNA changes of 14500 genes in 40 OSF and 4 normal buccal mucosa samples were used to identify novel biomarkers of OSF. Five candidate genes with the most differential changes were chosen for validation, and the correlation between clinico pathologic parameters of 66 OSF patients and the expression of each gene was assessed using immunohistochemistry. The microarray analysis showed that 661 genes were up-regulated (fold value>2) and 129 genes were down-regulated (fold value<0.5) in OSF (q<0.01).

The top three up-regulated genes [Loricrin, Cartilage oligomeric matrix protein(COMP), Cys-X-Cys ligand 9(CXCL9)]with the largest fold changes and top two down-regulated genes keratin 19(KRT19), cytochrome P450 3A5(CYP 3A5)]with the most significantly differential changes in OSF were chosen as candidate biomarkers. The expression of Loricrin showed statistically significant association with histological grade of OSF, P=0.03 with no detectable expression in the normal samples. However, 42 (63.6%) of 66 OSF cases exhibited intensively brown staining for Loricrin almost limited to the upper spinous epithelial layer and

sometimes in the keratinocyte layer. The staining was predominantly in the cytoplasm, whereas occasionally both cytoplasmic and nuclear immunoreactivity were observed. A significant increase in Loricrin expression was observed in OSF lesions in comparison with normal buccal mucosa specimens (NBM) specimens with over- expression seen frequently in the patients having the habit of chewing areca-nut for more than 4 years. The pilot study thus demonstrates 5 novel genes which might play important roles in the pathogenesis of OSF and can be clinically useful for early detection of OSF.[47]

Loricrin, acts as a protective barrier to protect from environmental hazards, and is one of the differentiation markers and the major protein of the cornified envelope of terminally differentiated keratinocyte . The abundance of Loricrin in keratinizing epithelia subject to considerable mechanical stress, such as human foreskin epidermis implies that expression of Loricrin is essential for the function of these tissues. Obviously, the coarse fibers of areca nut are the key mechanical stress to the epithelium of oral mucosa of OSF patients; thus, it is plausible to hypothesize to find corresponding increase of Loricrin expression in OSF samples. Although Loricrin is normally incorporated into the cornified envelope within 2 hours of synthesis in the differentiating keratinocyte, the enhanced expression of extractable Loricrin (not cornified envelope incorporated) in protein extracts from OSF, in this study, indicated that it was a protective response of the oral mucosa against the persistent mechanical irritation of areca nuts.

Loricrin in OSF was identified to be localized in the upper spinous epithelial layer and keratinocyte layer corresponding to its location in keratinizing epidermis, which could also provide support for the above mentioned hypothesis.

A significant difference of Loricrin staining between early stage and moderately advanced stage of OSF was found but not between moderately advanced stage and advanced stage. Which was reasoned to the limited number (n = 13) of advanced-stage OSF samples used in the study. Further it was postulated that loricrin could be a kind of protein with limited capacity against the persistently mechanical stress of areca nut in the advanced stage of OSF lesion as a result of the timing of loricrin expression during the development of mouse, when compared to that of the other epidermal differentiation markers like K14, K10 a and filaggrin and was found to correspond with human and other mammalian species development. [48]

Holbrook *et al* reported the expression of loricrin at around 7-8 wks gestation in the early intermediate cell layers of human epidermis. [48] Yoneda *et al* showed that endogenous loricrin was expressed by E16 in transgenic mice with RT-PCR. The histologic localization of loricrin with respect to the specific stages of mouse epidermal differentiation is expressed focally with the protein appearing in only a few granular cells while not being present in the periderm, very similar to the expression in the human foetal skin. The periderm which is the initial protective barrier of the developing epidermis, begins to get detatched around the time of E18, and the underlying epidermis has to be strong enough to take up the function of a strong barrier. The foetal stratum intermedium and stratum corneum provide a strong barrier by forming strong and flexible cell envelopes, strong cytoskeletal framework and by being impermeable to water. Loricrin by its property of being highly insoluble and by its shear mass helps as a protective barrier.[45]

Li N, Jian XC *et al* investigated the expression of loricrin and cytochrome P450 3A5 (CYP 3A5) in oral submucous fibrosis (OSF) to evaluate their roles in the defending ability of epithelium mucosae. On examining the specimens of 66 OSF and 14 normal buccal mucosa samples by immunohistochemistry, for the expression of loricrin they found that loricrin was overexpressed in 42 (63.6%) cases of OSF, and showed a significant difference only between the early and moderately stages of OSF ($P < 0.05$), but no clear difference between moderately and advanced stages ($P > 0.05$). Protein and mRNA expression of loricrin was detected by western blot and reverse transcriptase-PCR (RT-PCR), suggesting that the loricrin might play a vital role in the change of defending ability of epithelium as well as the pathopoiesis and carcinogenesis of OSF [49].

BE Kim *et al*, [50] in a study showed that TNF-α down regulates filaggrin and loricrin, and thereby affecting the epidermal barrier in certain skin disorders like psoriasis and atopic dermatitis.

A deficiency of filaggrin and loricrin in lesional and non-lesional psoriasis skin was demonstated using real time RT PCR and immunostaining and showed a similar picture on comparing normal skin and psoriatic skin. A decreased expression in the epidermal barrier proteins filaggrin and loricrin was noted indicating that the skin barrier defect seen in psoriatic conditions, are a result of deficiency in the epidermal barrier proteins. On examining the reason for the decreased expression of these barrier proteins it was found to be modulated by an increased expression of TNF –α, along with increase in some anti microbial peptides like HMB2 (2-Hydroxy-3-Methylbutanoic Acid) proteins which is the reason for decreased infections in Psoriasis inspite of the skin barrier defect. This

is not so in conditions like Atopic dermatitis where there is increased expression of Th2 cytokines which will inhibit the expression of loricrin and fillaggrin barrier proteins altogether with no expression of antibacterial peptides[50]

Loricrin mutation and associated diseases.

Loricrin, inspite of being the most predominant protein of the cornified envelope (CE) in keratinocytes, produces only modest skin phenotypes in case of loss or gain of function in murine models, but insertional mutations resulting in a frame shift in the C-terminal domain of loricrin produces the characteristic ichthyosis of loricrin keratoderma in mouse and man .

The unique heterozygous, insertion mutations in the loricrin gene have been found to underlie certain congenital skin abnormalities, the phenotypes of which vary considerably. Clinically, these patients can be diagnosed as suffering from an "Ichthyotic variant of Vohwinkel's syndrome", "Progressive symmetric erythrokeratoderma," or "congenital ichthyosiform erythroderma" born as a collodion baby. Common clinical features include hyperkeratosis of the palms and soles with digital constriction. Histologic characteristics include parakeratotic hyperkeratosis with hypergranulosis and nuclear accumulation of mutant loricrin. The unique mutations in the glycine-rich domain of the mutant loricrin form arginine-rich nuclear localization sequences (NLSs) that disrupt differentiation of keratinocytes. This group of unique genodermatoses caused by distinct loricrin mutations is collectively termed as loricrin keratoderma (LK).[51]

The basis for the loricrin keratoderma phenotype, was done by assessing the epidermal structure and stratum corneum function in a previously genotyped human loricrin keratoderma kindred, which showed an abnormal corneocyte fragility and basal permeability barrier function but an accelerated repair kinetics. Despite fragility, increased water loss occurred predominantly via extracellular domains, which could be due to the disorganized lamellar bilayers that are linked spatially to discontinuities of the CE. The accelerated barrier recovery was seen by amplified lamellar body secretion, while partial normalization of the CE in the outer stratum corneum correlated with persistence of abundant calcium in the extracellular spaces (positioned to activate transglutaminase-1).The results showed that the barrier abnormality in loricrin keratoderma is linked to a defective CE scaffold, resulting in increased extracellular permeability, which is similar to another scaffold disorder "lamellar ichthyosis", the only difference being the partial normalization of the CE scaffold in the outer stratum corneum in loricrin keratoderma.[44]

Three types of loricrin mutations have been detected in genomic DNA from five loricrin keratoderma families. All three mutations are heterozygous insertion mutations of a single nucleotide that result in a frameshift and a common downstream delayed termination codon. Immunohistochemisty further demonstrated that mutant loricrin was expressed in the loricrin keratoderma epidermis. The staining patterns were identical between the two Icthyocytic Variant of Vohwinkel syndrome patients and two Progressive symmetric erythrokeratoderma patients. The staining was localized in the differentiated keratinocytes in a predicted tissue and differentiation dependent manner and was

detected up to the cornified layer. Our results suggest that detection of the mutant loricrin in scraped horny layer by immunoblotting or immunohistochemistry might offer simple non-invasive screening tests for loricrin keratoderma.

Positive results have been elicited on testing these antibodies in a different family with Palmo plantar keratoderma (PPK). Subsequent DNA sequencing detected an insertion mutation of a single nucleotide in the loricrin gene.

As for the patho- mechanisms of dominantly inherited disorders, some are explained as dominant negative effects of mutant molecules and are attributed to haploinsufficiency. Although loricrin constitutes about 70% of CE in normal skin, [37,40,43] the phenotype of loricrin null mice was just temporary and lasted only 4–5 d after birth which suggests that loricrin keratoderma is not caused by haploinsufficiency of loricrin but by some dominant negative effects of the mutant loricrin. The nature of these dominant negative effects remains unknown, but the possibility that the mutant loricrin disrupts assembly of CE is considered [52,53]

Because of the frame shift mutations, mutant molecules lack the C-terminal glutamine-lysine rich domain essential for cross linking CE of the LK cornified cells and were immunoreactive to involucrin antibodies, but showed very little immunoreactivity to loricrin antibodies. This is contrary to the normal CE that was positive to loricrin but negative to involucrin. Wild-type loricrin was detected along the CE in the granular cells of loricrin keratoderma, and loricrin epitopes on the CE of cornified cells were retrieved by protease digestion of the sections. This implies that normal loricrin is cross linked into CE of loricrin keratoderma, but its epitopes are masked by extensive further cross linking with other molecules such

as involucrin. Involucrin is one of the early constituents of CE with their epitopes normally masked when the CE is matured by later cross linking of loricrin and other molecules By contrast, involucrin cross linking seems to continue after loricrin is cross linked and masks loricrin epitopes in LK. Because mutant loricrin is expressed within the nuclei and not associated with cell membranes or CE, it is unlikely that the mutant loricrin is cross linked into CE directly interfering with its formation. But it could be speculated that mutant loricrin deranges keratinocyte terminal differentiation by some other mechanisms.[52] Nuclear localization of mutant loricrin suggests that it exerts dominant negative effects through interactions with nuclear proteins and/or nucleic acids. The Arginine-rich sequences of mutant loricrin show homology to a highly basic nuclear protein, called protamines which interact with DNA on their Arginine clusters. Alternatively initial association with nucleoli might indicate a pathologic role of the mutant loricrin upon nucleolar functions.

In the LK epidermis, nucleoli from the basal to the lower granular layer were not apparently different from those in normal skin, but those in the upper layer were distinct. There was deposition of mutant loricrin within and around the nucleoli. Localization of mutant loricrin in the dense fibrillar component and granular component of the cells with a high level of ribosomal biosynthesis suggests interaction between mutant loricrin and rRNA or rRNA binding proteins. Recent evidence also suggests that nucleolus is a preferred intracellular target for apoptotic events. Terminal differentiation of keratinocytes is a typical example of programmed cell death . Mutant loricrin might derange the keratinocyte differentiation/cell death pathway by affecting nucleolus as a target of apoptotic

cellular changes, which explains the delay of the cell death process in loricrin keratoderma epidermis in the stage where nuclear DNA is fragmented [53] Alternatively mutant loricrin might disturb other functions of nucleolus that include non ribosomal RNA processing and growth factor signal transduction [50,51]

The reason for limited distribution of skin lesions in loricrin keratoderma patients despite the generalized expression of the mutant loricrin is obscure at this moment. In the non lesional skin mutant loricrin-expressing cells were just one cell layer thick and were to cornify in the next cell layer. It might be that, if mutant loricrin is expressed in a very late stage of terminal differentiation where nucleolus is no more active, the mutant loricrin does not hamper the completion of differentiation.[37,52]

In the palmo-plantar skin loricrin-expressing cells are several layers thick. When the cells start to express mutant loricrin, they still have a long way to go with late keratinisation processes that are more likely to be affected. But the reason for variation in the extra-palmoplantar lesions in “Progresive symmetric erythro keratoderma” and “Ichthyocytic variant of vohwinkel syndrome” is obscure.Small proteins with molecular mass less than 40–60 kDa can cross the nuclear pore complex by diffusion loricrin is a small molecule (26 kDa in human and 38 kDa in mouse) and localized in both the cytoplasm and nucleoplasm .The mechanisms for nuclear translocation and specific association with nucleolus of mutant loricrin remain unknown at present. The sequences of mutant loricrin rich in arginine might function as consensus bipartite nuclear localization signals. A nuclear localization signal *or* sequence (NLS) is an amino acid sequence which 'tags' a

protein for import into the cell nucleus by nuclear transport. Typically, this signal consists of one or more short sequences of positively charged lysines or arginines exposed on the protein surface. Different nuclear localized proteins may share the same nuclear localization signal (NLS).A pepta-peptide (RRQRR), which has been implicated as part of a nucleolar targeting element in signal recognition particles , is present in the mutant loricrin and this might be functional. These possibilities have to be tested in future studies.[37]

Given the various consequential implications of the mutation in loricrin in skin conditions like loricrin keratoderma , this could be correlated with the condition of OSF and other conditions exhibiting hyperkeratosis like leukoplakia wherein the expression of this loricrin in a mutant form could be appreciated using immunohistochemistry and could result in a proper evaluation of the diseased state.

Results

PATIENT CHARACTERISTICS:

The study population consisted of (n= 73) cases taken from archival blocks, They were segregated into three groups with Group I (n=11) comprising of clinically normal appearing mucosa specimen samples, Group –II comprising of (n= 32) Hyperkeratosis samples [Frictional keratosis(n=21) and epithelial dysplastic lesions (n=11)] and Group III comprised of (n=30) oral submucous fibrosis (OSF) specimen samples.

SITE:

All the samples were archival blocks with tissues taken from buccal mucosa. Of the these samples, 5 samples were taken from the retromolar region(1 case was grouped under Group II, and 4 cases under Group-I) and 1 sample was taken from the right lateral border of tongue. (grouped under Group-II). The samples were evaluated for the expression of loricrin and any staining was considered positive.

GENDER DISTRIBUTION:

The samples were predominantly taken from male patients with the 81.8% of the patients being male in the normal mucosa samples, 93.8% and 86.7% respectively in hyperkeratosis and OSF samples. The female population among the study groups were distributed as 11.0%, 6.3% and 13.3% respectively in groups I, II and III. The P value 0.475 showed that there was no significant relation between gender and the study groups.(Table-1;Graph-1)

STAINING POSITIVITY:

Overall distribution of positive loricrin expression among the three groups.

Histopathological evaluation of anti bodies directed against loricrin revealed positivity in all three groups.

Group I Normal mucosa (n=11)showed negative staining in all the samples except 2 cases. ie.(n=9) 81.8% stained negative, while (n= 2) 18.2% showed mild positivity.

Group II Hyperkeratosis (n= 32), showed positive staining in n= 27 with 84.4% and n=5 stained negative with 15.6%.

Group III OSF specimens , 66.7% (n = 20) showed positive staining and 33.3%. (n=10) showed negative staining. The P value 0.000 showed that there was a statistically significant relation between the positive expression of loricrin and the study groups**.** (Table-2; Graph-2)

STAINING INTENSITY:

Distribution of staining index of loricrin among the 3 groups were analysed and the intensity of staining was graded as nil, mild, moderate and intense .

Normal mucosa showed a greater percentage 81.8% (n=9) of negative expression. While those cases which showed positivity were of mild intensity and comprised of about 11.0% (n=2).

Hyperkeratosis cases revealed a majority of the cases taking up staining with 25.0% (n=8) taking mild stains, 43.8% (n=14) taking up moderate stains and 15.6% (n=5) taking up intense staining. 15.6% (n=5) did not take up any stains .

Among OSF samples,33.3% (n=10) of the cases had not taken up staining. Mild and intense staining were appreciable equally with 16.7% (n=5) of the cases. Moderate staining is seen in about 33.3%.(n=10). P- value is 0.006 and is

significantly higher on comparing the distribution of staining index of loricrin among the three groups. (Table-3; Graph-3)

Distribution of staining index in the stratum spinosum

The staining index in three stratas of epithelium namely stratum spinosum, stratum granulosum and stratum corneum was analysed.

Normal mucosa and hyperkeratosis samples did not show any staining in their stratum spinosal layer. However the stratum spinosum in the OSF samples showed different gradations of staining intensities with no staining shown by 83.3% (n=25) cases, while mild and intense staining was observed in equal number of cases n=2 each with 16.7%. (n=3) 33.3% of the cases showed moderate staining. The results were not statistically significant (P=0.261) **(**Table 4;Graph-4)

Distribution of staining index in the stratum granulosum

On comparing the degrees of staining taken up by the stratum granulosum in the three groups, Normal mucosa specimens showed only a mild positive staining, taken up by one case n= 1 (9.1%). Hyperkeratotic specimens showed the most staining in this layer, while n =5 (15.6%) cases of the 32 samples showed no positivity, n=8 (25.0%), n=14 (43.8%) and n=5 (15.6%)cases showed mild, moderate and intense staining respectively. OSF on the other hand had 12 cases which did not take up staining in this layer, but showed an increase in the number of cases exhibiting an intense staining reaction in n= 5 (16.7%) cases. Moderate staining was seen in 9 (30.0%) followed by mild staining in n=4 (13.3%) of the cases. The P value of 0.002 is found to be statistically significant on comparison of the staining intensity evaluated in stratum granulosum among the three groups. The difference was statistically significant (P = 0.002) (Table 5; Graph 5**)**

Distribution of staining index in the stratum Corneum

No staining was observed in the case of normal mucosa. Hyperkeratosis specimens shows no staining in n=28 (87.5%)cases while moderate and intense staining were exhibited by 2 cases each with a percentage of 6.3%. Of the OSF specimens n= 26 (86.7%) cases showed no staining in the stratum corneum with n=1(3.3%) each showing mild and intense staining. Moderate staining was seen in 2 cases giving a percentage of n=2 6.7%. the difference was not statistically significant (P =0.786)(Table 6; Graph 6)

Distribution of habits among the three groups.

Assessment of the presence of habit history (chewing, smoking and drinking) revealed that, out of the total 73 cases in group I ,II and III , 55 had habits and 18 had no habits.

In Group I (Normal mucosa) one (n=1; 9.1%) person had habit history and ten (n= 10; 90.9%) others had no habits. while in Group II (Hyperkeratosis) twenty six (n=26; 81.3%) were to found to have habits : and six (n=6; 18.8%) had no habits.

In Group III OSF 28 (93.3%) had habits and 2 (6.7%) had no habits.

Among the groups OSF shows the maximum percentage of 50.9% of patients having a habit history followed by hyperkeratosis with 47.3% and normal mucosa (1.8%).The difference among the groups were statistically significant.(p = 0.000) (Table-7 ;Graph-7)

Distribution of chewing habit among the groups.

Analyzing the distribution of chewing habit alone among the groups that it was seen in only one case among the normal mucosa specimens. However 25 cases out of the 32 hyperkeratosis specimens had the habit of chewing giving a percentage of 78.1%.In the OSF group, 28 cases (93.3 %) had the habit of chewing. The difference was statistically significant.

(P = 0.000)(Table 8 ; Graph 8)

Comparing habit of chewing within the groups

Within the groups the habit was predominantly seen in OSF with about 51.9% (n= 28) of them having the chewing habit. 25 hyperkeratosis cases (46.3%) had the habit when comparing it with normal mucosa showed only 1(1.9%) case with the habit of chewing. The habit of chewing among groups was statistically significant (p =0.000)(Table-9 ; Graph 9)

Comparing positive loricrin expression within hyperkeratosis.

On comparing the positivity of loricrin expression within the hyperkeratosis group, the physically induced hyperkeratosis group showed 90.5% (n=19) positivity while the chemically induced group showed 72.7% (n=8) of cases with positive loricrin expression. The difference in expression is not statistically significant. (P=0.209) Table-10; Graph-10)

Comparison of staining intensity within hyperkeratosis

Comparison between the different intensities within the hyperkeratosis groups showed that the physically induced hyperkeratosis group had n=9 cases

which showed no staining, n=2 cases (18.2%) showed mild positivity while moderate and intense staining was not observed in any cases. The chemically induced cases showed n=5 (15.6%) case with no staining and n=8 cases (25 .0%) showing mild cases and n=14(43.8%) showed moderate staining and n=5 cases(15.6%) showed intense staining. The staining intensities were not statistically significant. (P=0.563). (The results of kappa analysis was 0.4)

Tables and Graphs

TABLE 1

DISTRIBUTION OF GENDER IN THE STUDY GROUPS (n=73)

GROUPS	TOTAL	GENDER		P VALUE
		MALE	FEMALE	
Group-I Normal mucosa	n =11	9 (81.8%)	2 (11.0%)	
Group–II Hyperkeratosis	n = 32	30 (93.8%)	2 (6.3%)	0.475
Group-III OSF	n =11	26 (86.7%)	4 (13.3%)	

GRAPH 1

DISTRIBUTION OF GENDER IN THE STUDY GROUPS

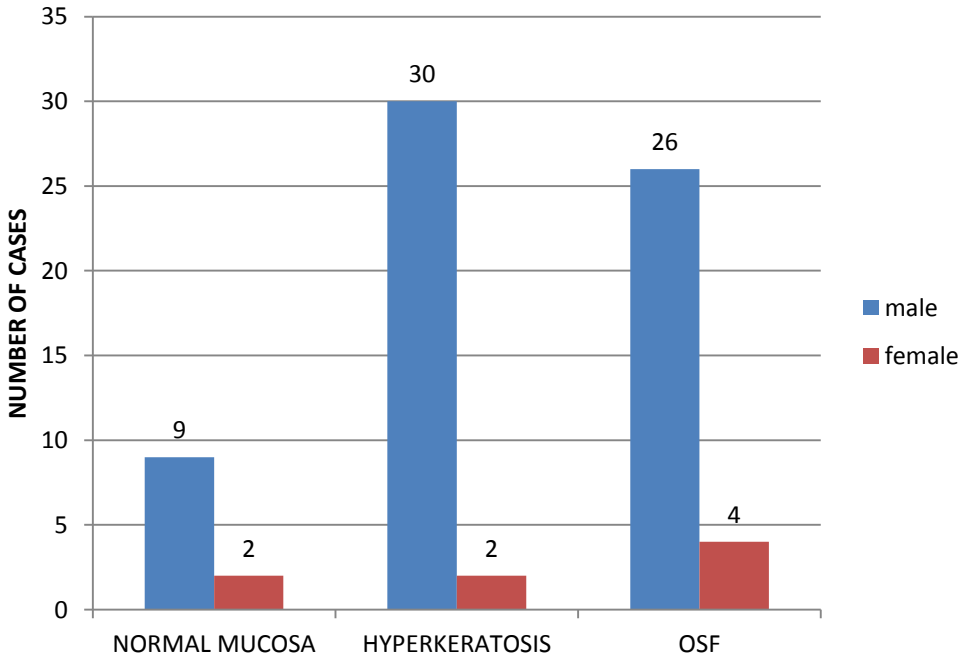


TABLE 2

DISTRIBUTION OF POSITIVE LORICRIN EXPRESSION IN THE STUDY GROUPS (n=73)

GROUPS		POSITIVITY		P VALUE
		POSITIVE	NEGATIVE	
Group-I Normal mucosa	n = 11	2(18.2%)	9(81.8%)	0.000*
Group-II Hyperkeratosis	n = 32	27(84.4%)	5(15.6%)	
Group-III OSF	n =30	20(66.7%)	10(33.3%)	

* Statistically significant at 5% level, P<0.05

GRAPH 2

DISTRIBUTION OF POSITIVE LORICRIN EXPRESSION IN THE STUDY GROUPS (N=73)

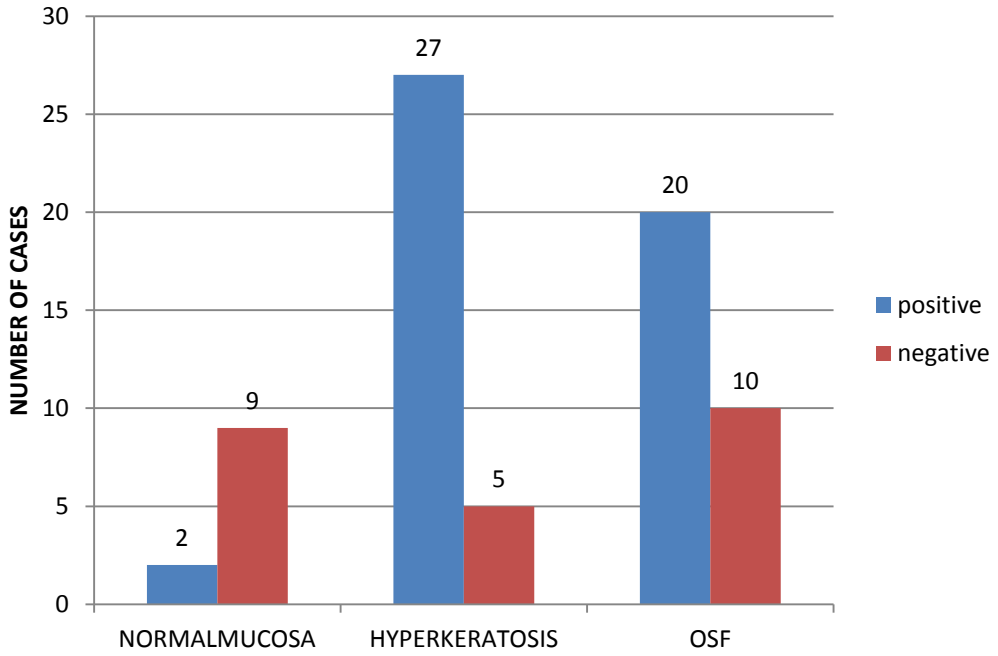


TABLE 3

OVERALL DISTRIBUTION OF THE STAINING INDEX AMONG THE THREE GROUPS

GROUP		INTENSITY GRADING				P VALUE
		NIL	MILD	MODERATE	INTENSE	
Group-I Normal mucosa	n =11	9(81.8%)	2 (18.2%)	0 (0%)	0 (0%)	0.006
Group-II Hyperkeratosis	n =32	5 (15.6%)	8 (25.0%)	14 (43.8%)	5 (15.6%)	
GroupIII OSF	n =30	10(33.3%)	5(16.7%)	10(33.3%)	5(16.7%)	

GRAPH 3

OVERALL DISTRIBUTION OF THE STAINING INDEX AMONG THE THREE GROUPS

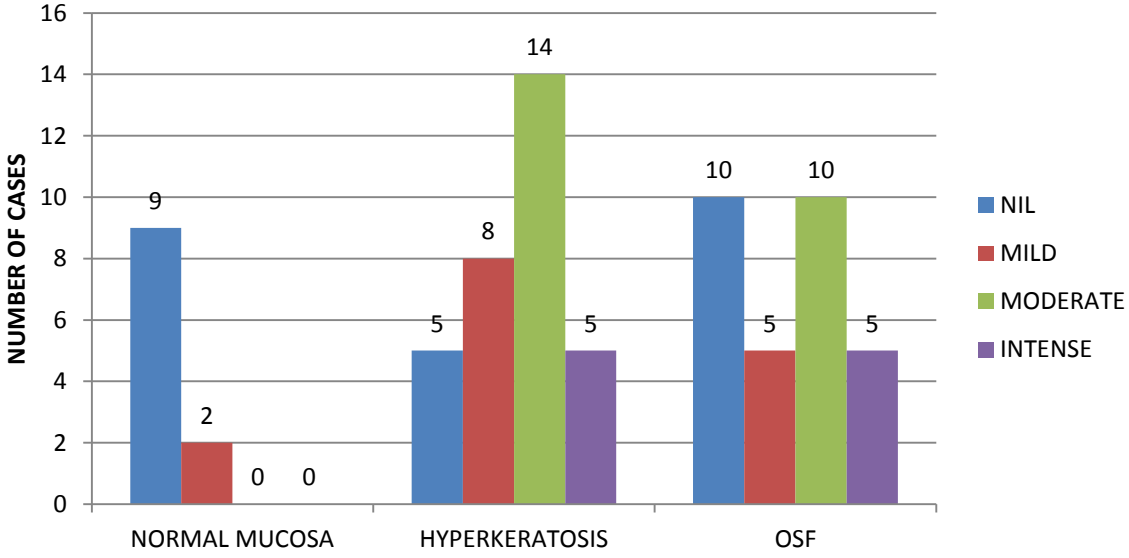


TABLE 4

DISTRIBUTION OF STAINING INDEX IN THE STRATUM SPINOSUM

GROUPS		STRATUM SPINOSUM				P VALUE
		NIL	MILD	MODERATE	INTENSE	
Group-I Normal mucosa	n =11	11 (100.%)	0 (0%)	0 (0%)	0 (0%)	0.261
Group-II Hyperkeratosis	n =32	32 (100.%)	0 (0%)	0 (0%)	0 (0%)	
Group-III OSF	n =30	25 (83.3%)	2(6.7%)	1(3.3%)	2(6.7%)	

GRAPH 4

DISTRIBUTION OF STAINING INDEX IN THE STRATUM SPINOSUM

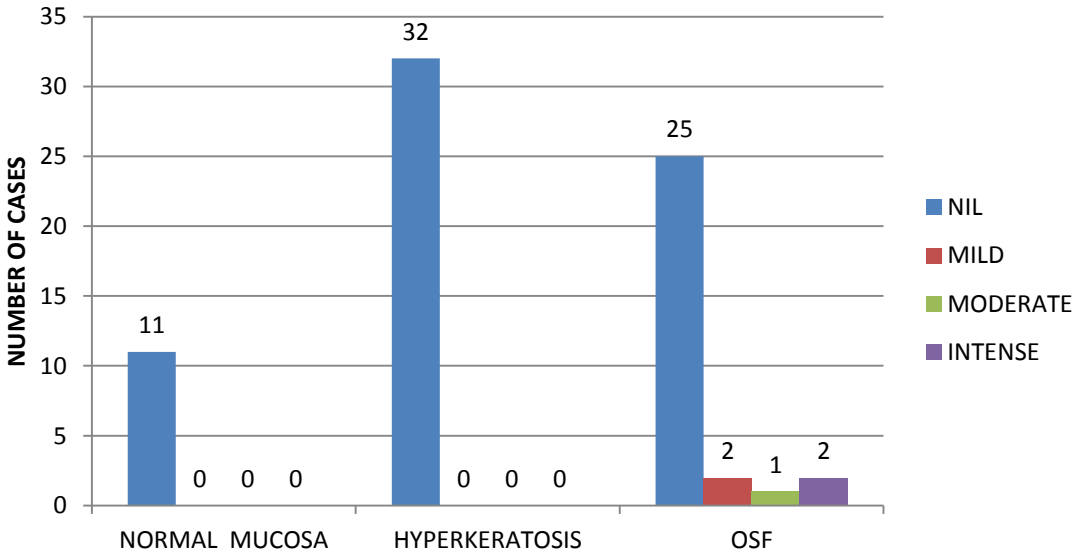


TABLE 5

DISTRIBUTION OF STAINING INDEX IN STRATUM GRANULOSUM

GROUPS		STRATUM GRANULOSUM				P VALUE
		NIL	MILD	MODERATE	INTENSE	
Group-I Normal mucosa	n =11	9(81.2%)	2(18.2%)	0 (0%)	0 (0%)	0.002*
Group-II Hyperkeratosis	n=32	5(15.6%)	8(25.0%)	14(43.8%)	5(15.6%)	
Group-III OSF	n =30	12(40.0%)	4(13.3%)	9(30.0%)	5(16.7%)	

* Statistically significant at 5% level, P<0.05

GRAPH 5

DISTRIBUTION OF STAINING INDEX IN THE STRATUM GRANULOSUM

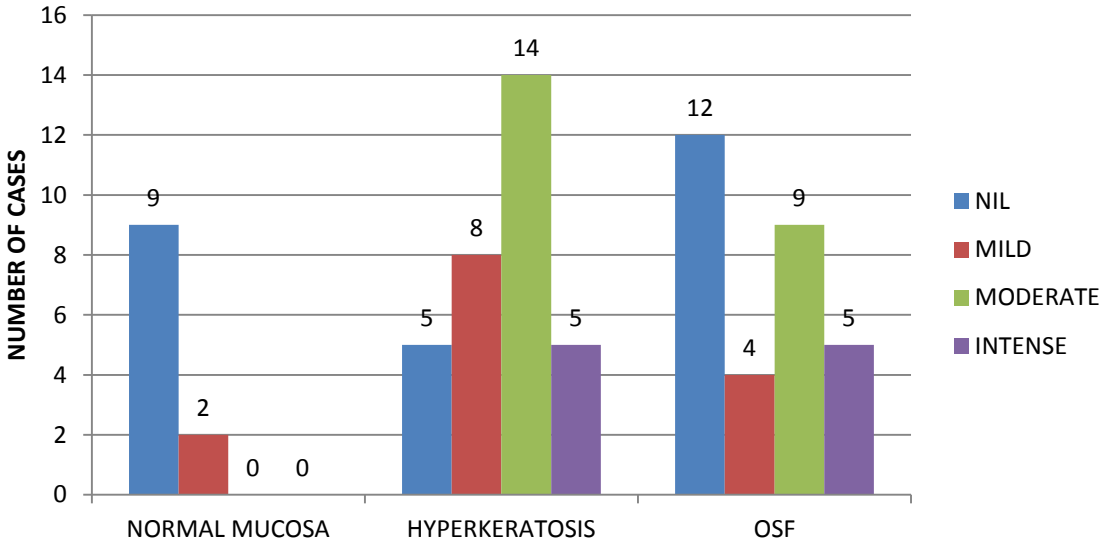


TABLE 6

DISTRIBUTION OF STAINING INDEX IN THE STRATUM CORNEUM

GROUPS		STRATUM CORNEUM				P VALUE
		NIL	MILD	MODERATE	INTENSE	
Group-I Normal mucosa	n =11	11(100.0%)	0 (0%)	0 (0%)	0 (0%)	0.786
Group-II Hyperkeratosis	n =32	28(87.6%)	0 (0%)	2 (6.2%)	2 (6.2%)	
Group-III OSF	n =30	26(86.7%)	1(3.3%)	2(6.7%)	1(3.3%)	

GRAPH 6

DISTRIBUTION OF STAINING INDEX IN THE STRATUM CORNEUM

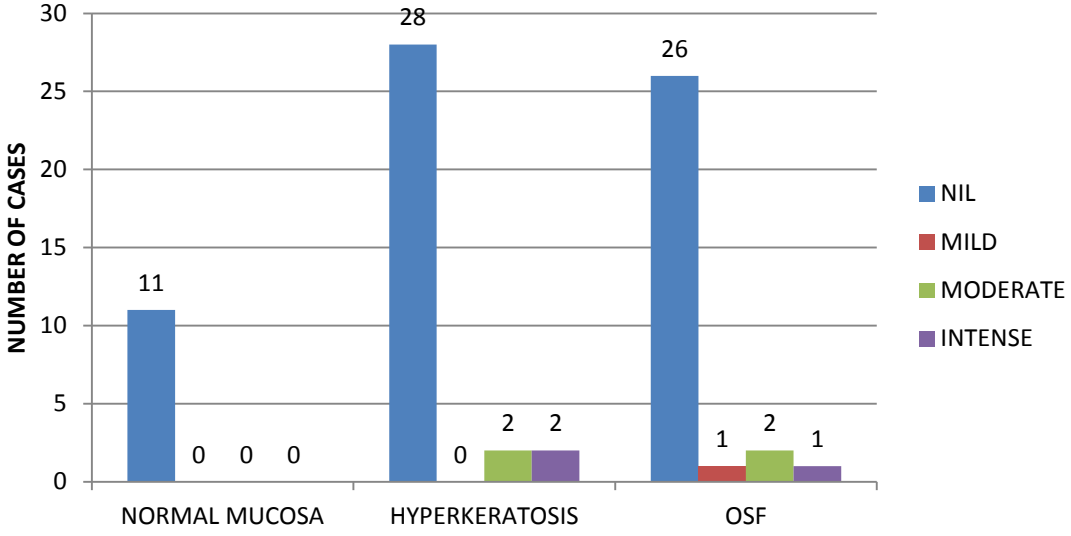


TABLE 7

DISTRIBUTION OF HABITS AMONG THE THREE GROUPS

GROUPS		HABITS		P VALUE
		PRESENT	ABSENT	
Group-I Normal mucosa	n =11	1(9.1%)	10(90.9%)	0 .000*
GroupII Hyperkeratosis	n = 32	26(81.3%)	6(18.8%)	
Group-III OSF	n = 30	28(93.3%)	2(6.7%)	

* Statistically significant at 5% level, P<0.05

GRAPH 7

DISTRIBUTION OF HABITS AMONG THE THREE GROUPS

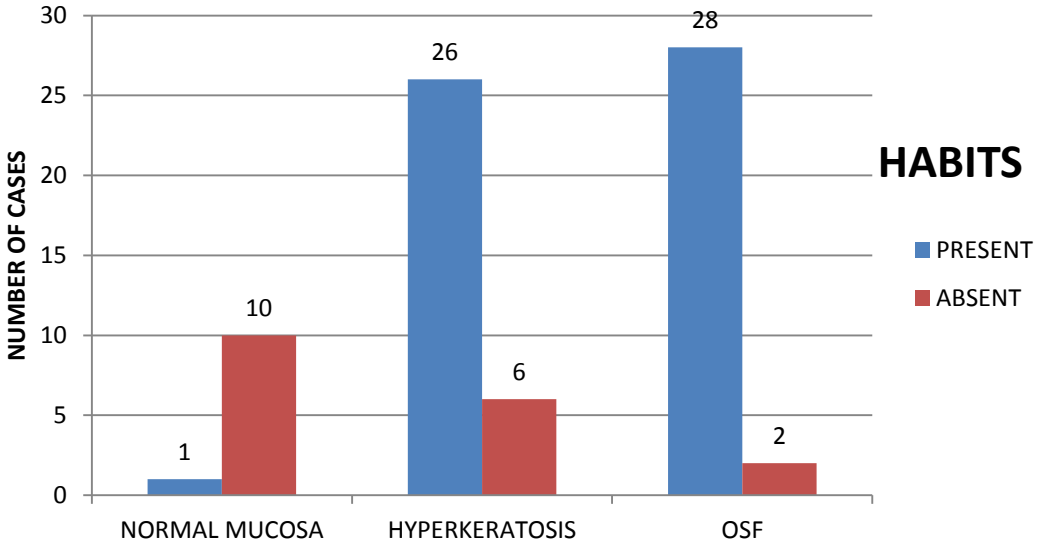


TABLE 8

DISTRIBUTION OF CHEWING HABIT IN EACH GROUP.

GROUPS		CHEWING HABIT		P VALUE
		PRESENT	ABSENT	
Group-I Normal mucosa	n =11	1(9.1%)	10(90.9%)	0.000*
Group-II Hyperkeratosis	n = 32	25(78.1%)	7(21.9%)	
Group-III OSF	n = 30	28(93.3%)	2(6.7%)	

* Statistically significant at 5% level, P<0.05

GRAPH 8

DISTRIBUTION OF CHEWING HABIT IN EACH GROUP.

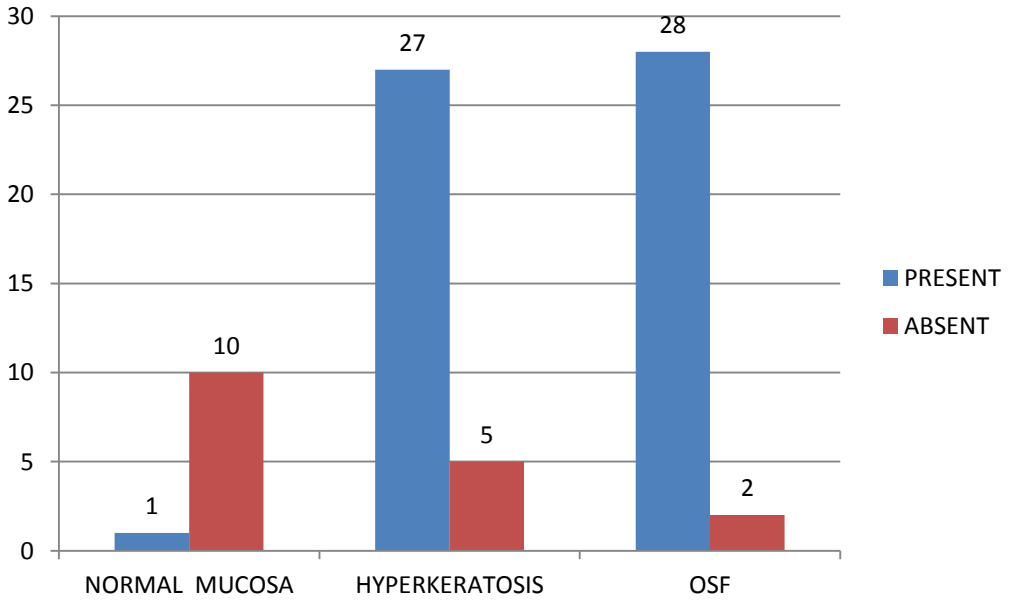


TABLE 9

DISTRIBUTION OF FREQUENCY WITHIN THE CHEWING HABIT IN THE GROUPS

GROUPS		CHEWING HABIT		P VALUE
		PRESENT	ABSENT	
Group-I Normal mucosa	n =11	1(1.9%)	10(52.6%)	0.000*
Group-II Hyperkeratosis	n =32	25(46.3%)	7(36.8%)	
Group-III OSF	n = 30	28(51.9%)	2(10.5%)	

* Statistically significant at 5% level, P<0.05

GRAPH 9

DISTRIBUTION OF FREQUENCY WITHIN THE CHEWING HABIT IN THE GROUPS

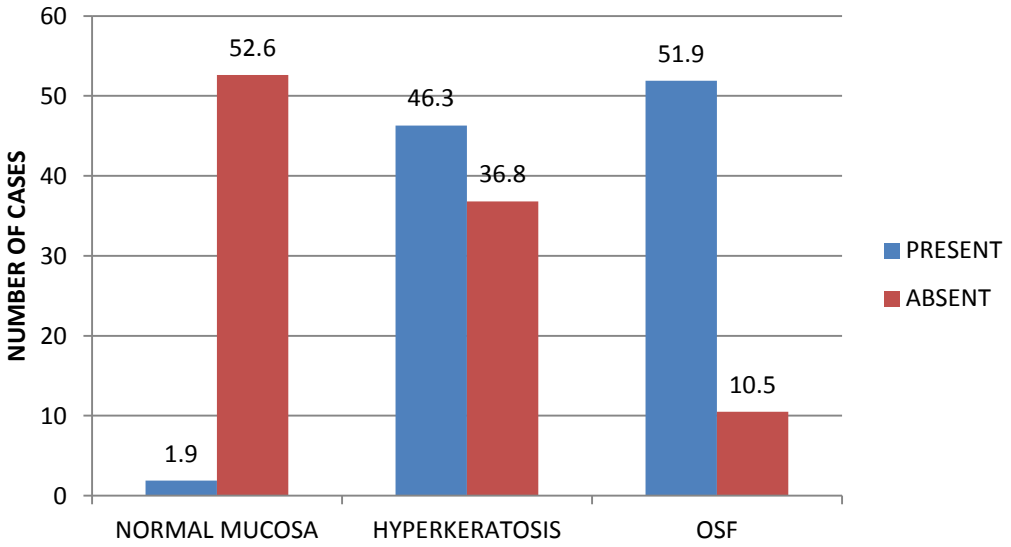


TABLE-10

DISTRIBUTION OF POSITIVE LORICRIN EXPRESSION WITHIN THE HYPERKERATOSIS GROUP.

GROUPS		POSITIVITY		P VALUE
HYPERKERATOSIS		**POSITIVE**	**NEGATIVE**	
PHYSICALLY INDUCED	n = 21	19(90.5%)	2(9.5%)	0.209
CHEMICALLY INDUCED	n = 11	8(72.7%)	3(27.3%)	

GRAPH-10

DISTRIBUTION OF POSITIVE LORICRIN EXPRESSION WITHIN THE HYPERKERATOSIS GROUP.

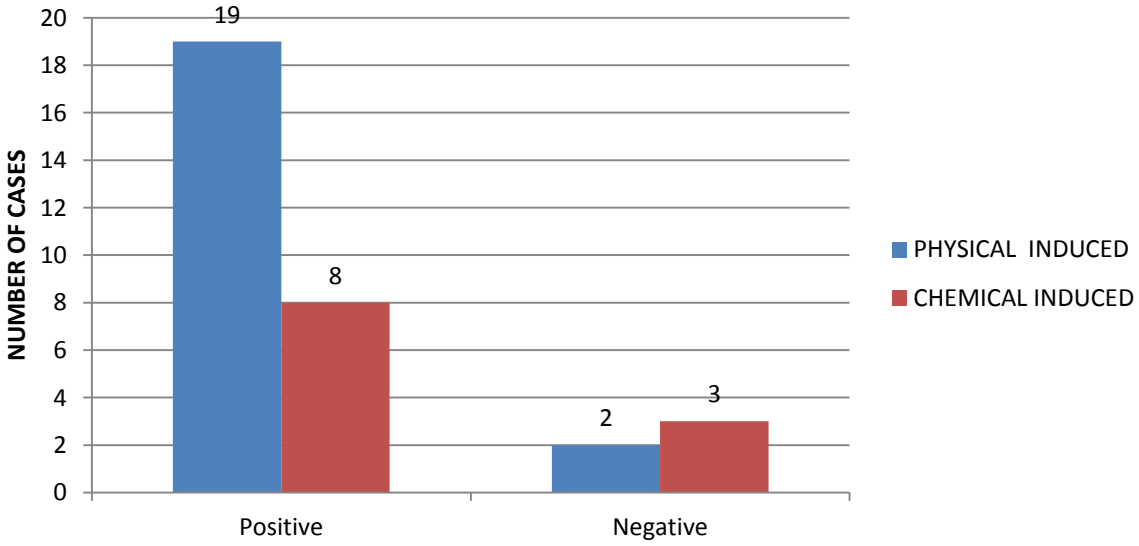


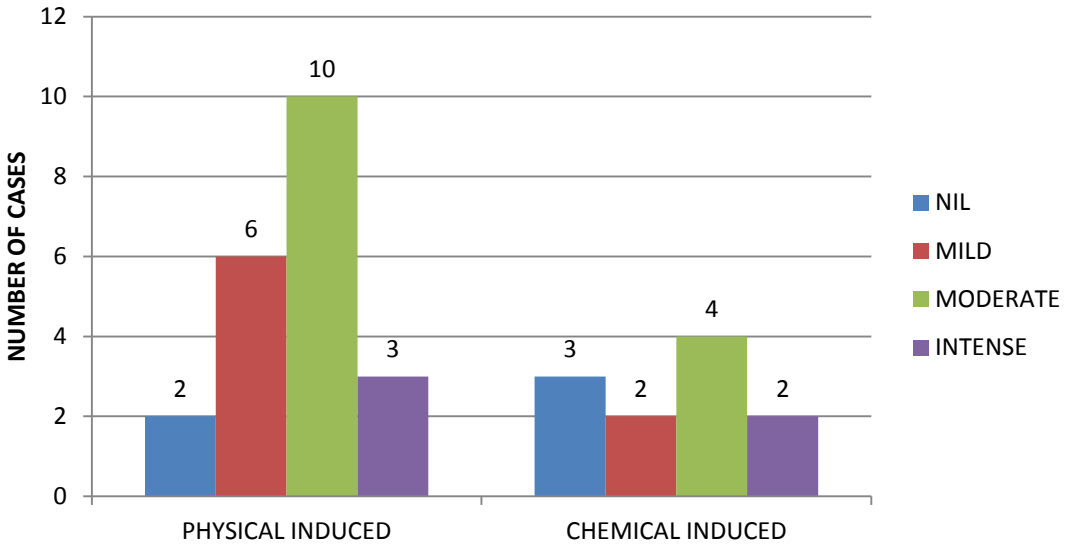
TABLE-11

DISTRIBUTION OF STAINING INDEX WITHIN HYPERKERATOSIS

HYPERKERATOSIS	INTENSITY GRADING				P VALUE
	NIL	MILD	MODERATE	INTENSE	
PHYSICAL INDUCED	9(81.8%)	2 (18.2%)	0 (0%)	0 (0%)	0.563
CHEMICAL INDUCED	5 (15.6%)	8 (25.0%)	14 (43.8%)	5 (15.6%)	

GRAPH-11

DISTRIBUTION OF STAINING INDEX WITHIN HYPERKERATOSIS



Figures

ARMAMENTARIUM

Fig.1

Fig 2:

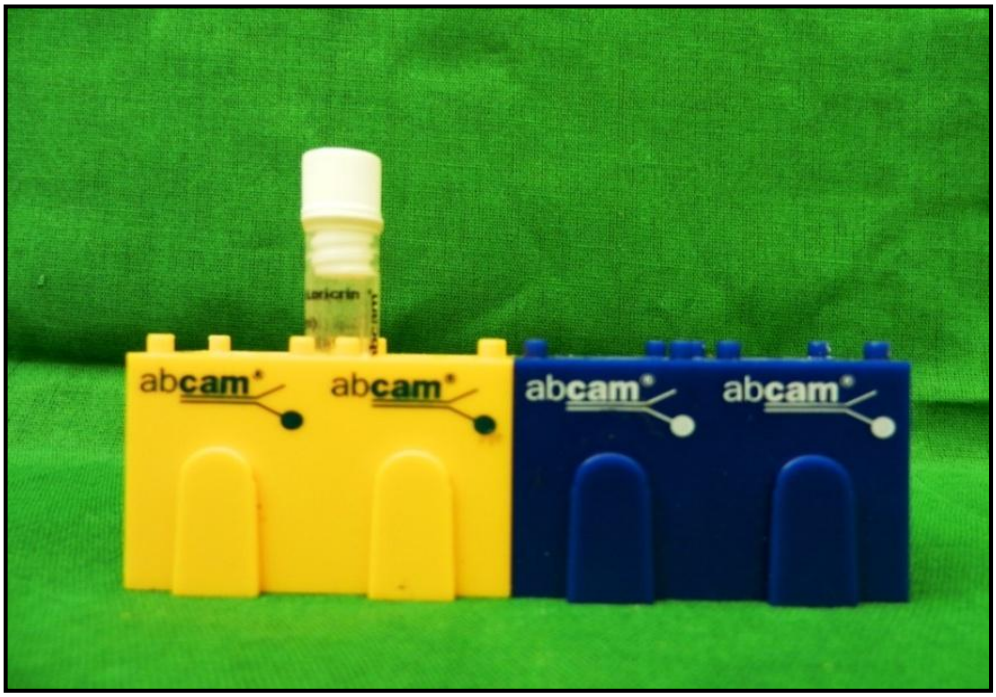


Fig 3: Primary Antibody

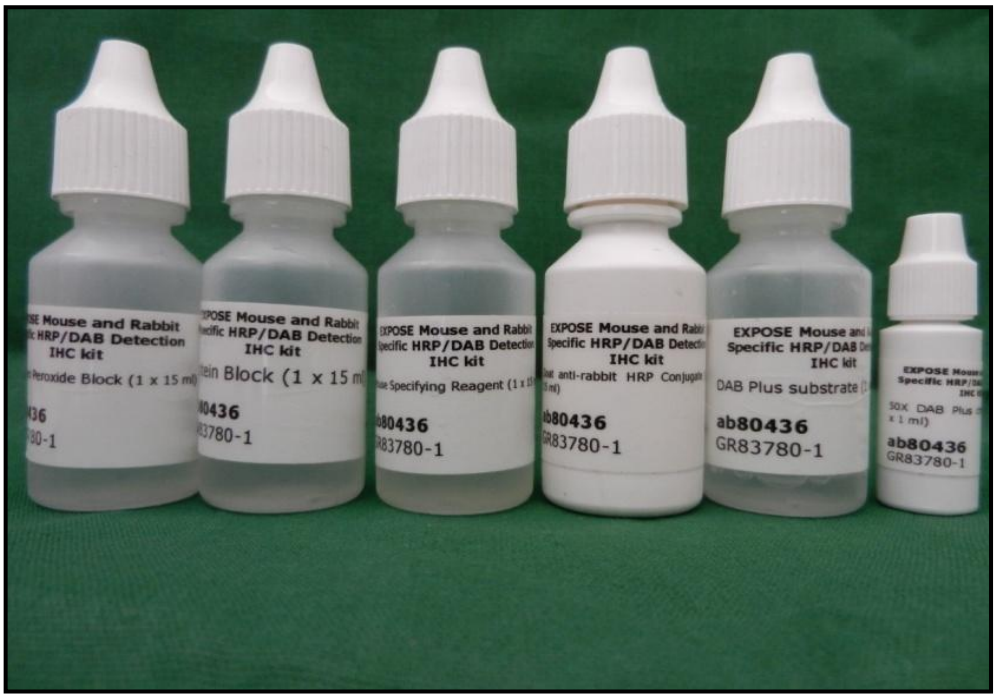


Fig 4: Secondary antibody

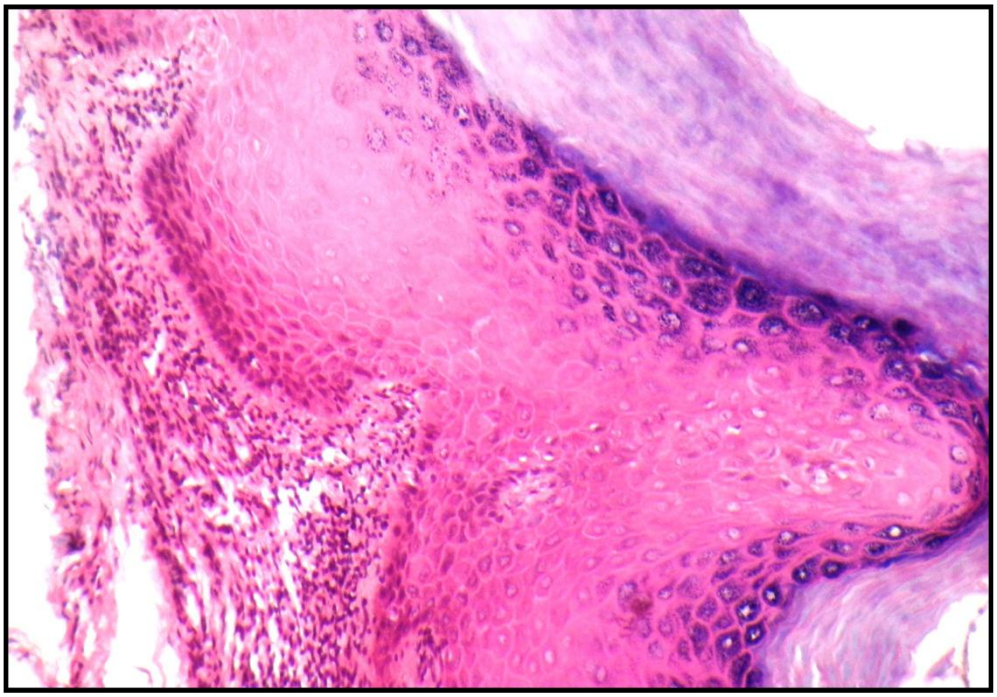


Fig 5: Hyperkeratosis; Haematoxylin and Eosin staining; (X10)

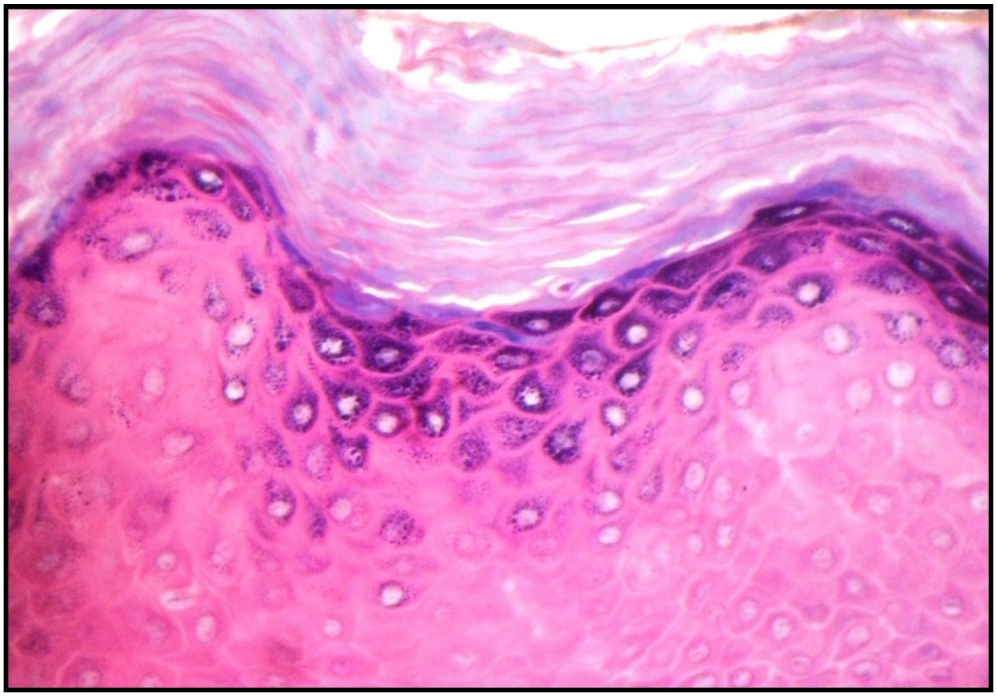


Fig 6: Hyperkeratosis; Haematoxylin and Eosin staining; (X 40)

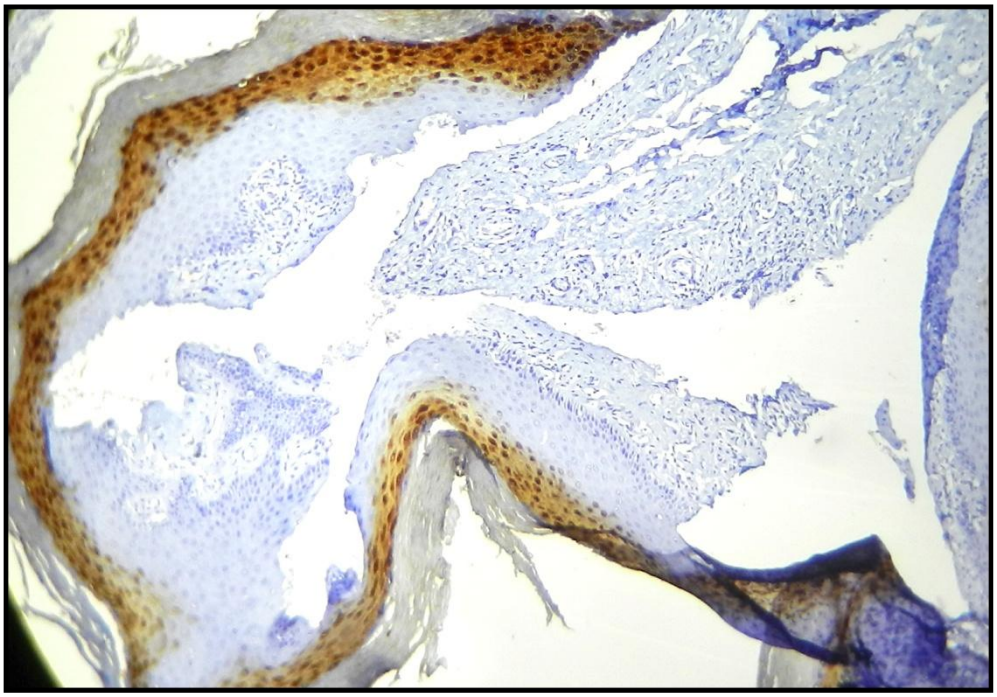


Fig 7: Hyperkeratosis without epithelial dysplasia showing loricrin positivity in the stratum granulosum by IHC (X10)

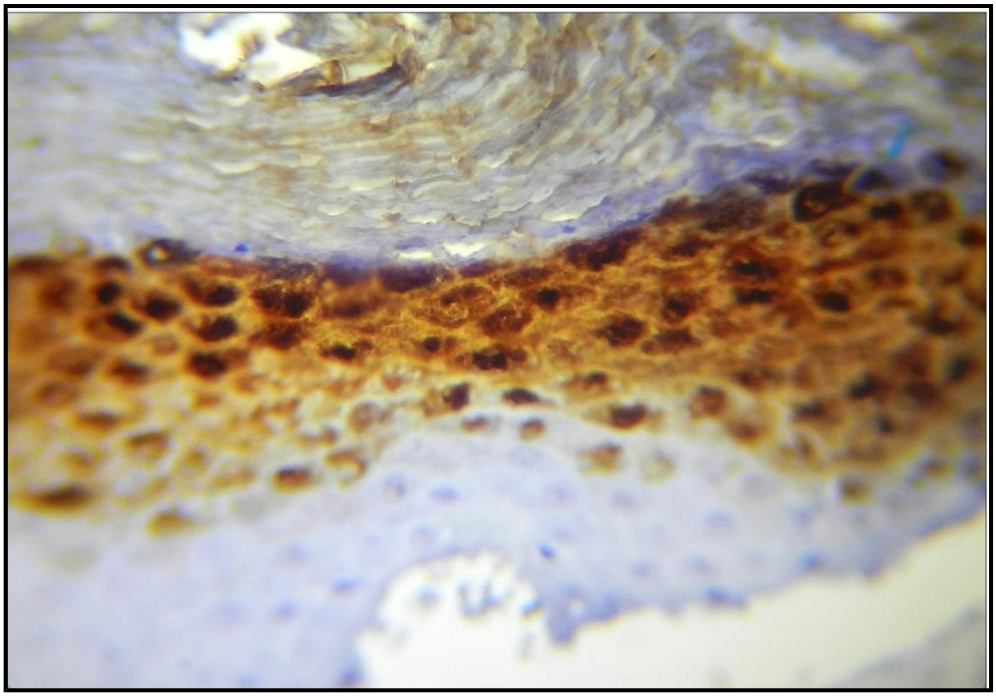


Fig 8: Hyperkeratosis without epithelial dysplasia showing loricrin positivity in the stratum granulosum by IHC (X40)

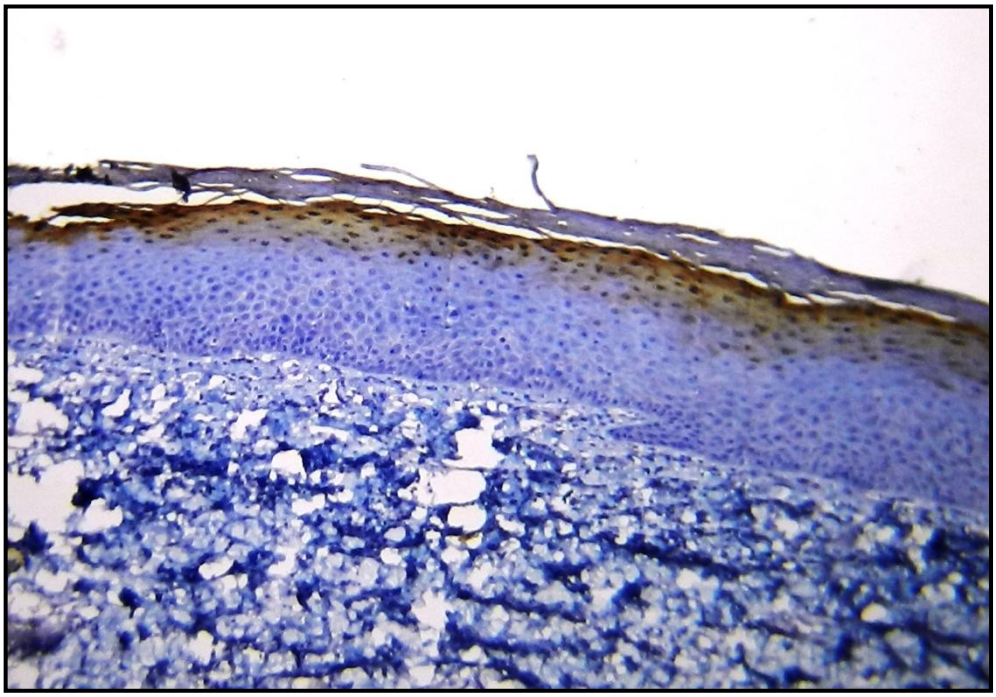


Fig 9 :Hyperkeratosis s with epithelial dysplasia showing loricrin positivity in the granular layer by IHC (X10)

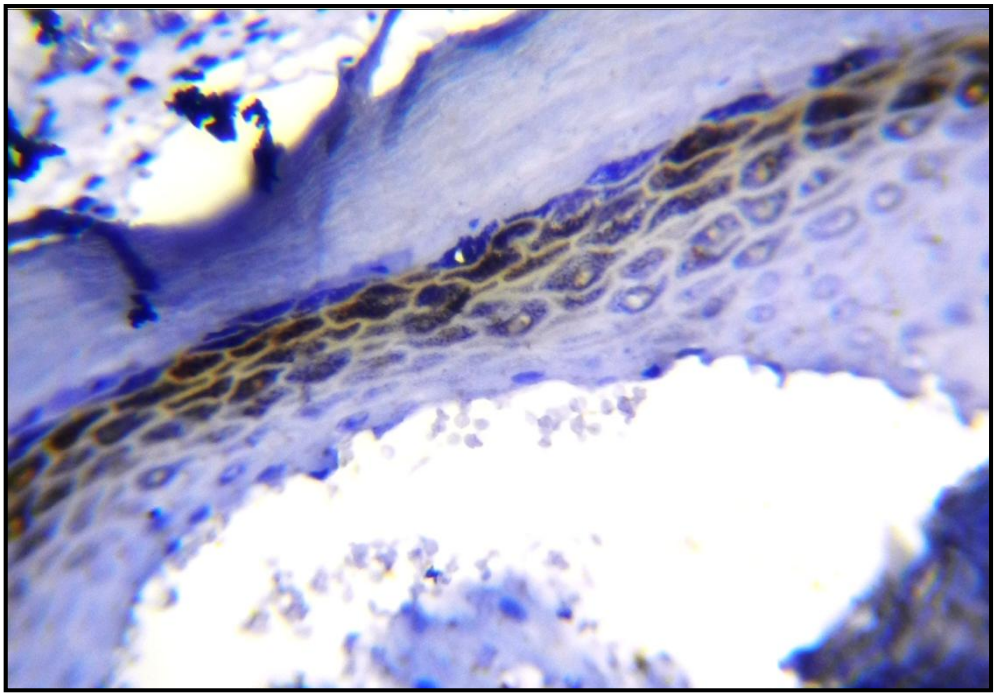


Fig 10: Hyperkeratosis with epithelial dysplasia showing loricrin positivity(X40)

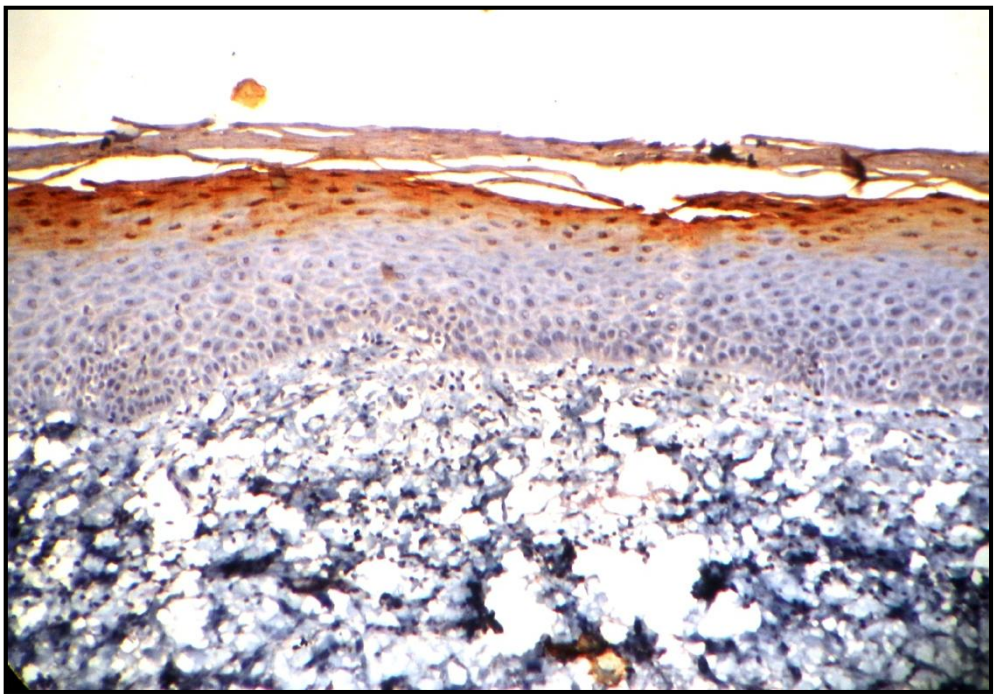


Fig 11 :Oral submucous fibrosis showing loricrin positivity in the granular layer by IHC (X10)

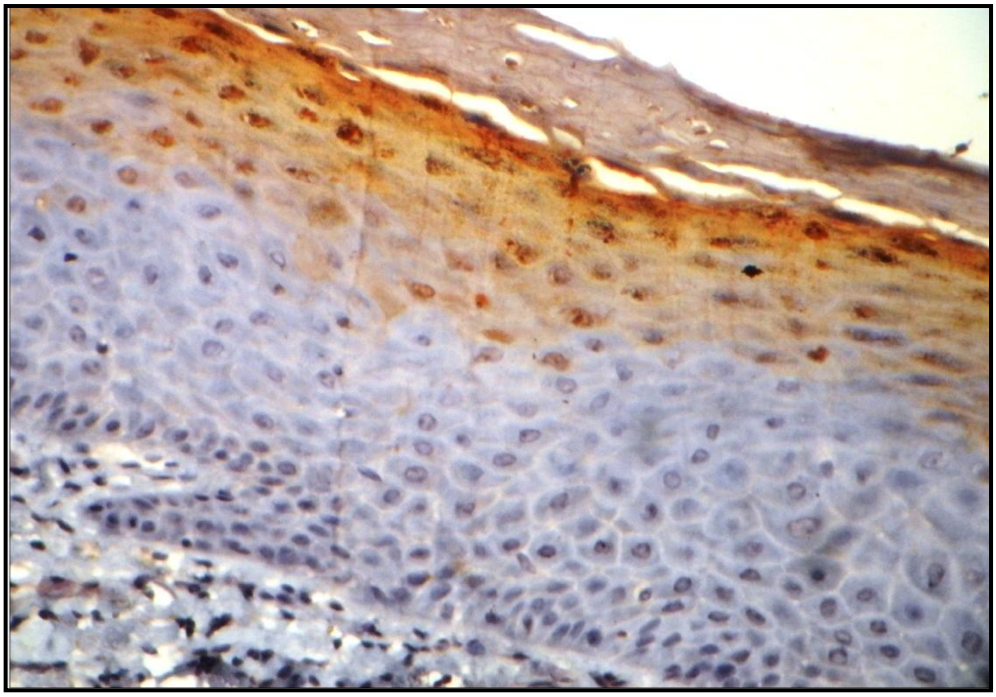


Fig 12: Oral submucous fibrosis samples showing loricrin positivity in the granular layer by IHC. (X40)

Discussion

The oral mucosa is exposed to a lot of stress, by both physical and chemical agents. To establish its integral role as an effective barrier with properties of flexibility and selective permeability, the mucosa of the oral cavity has been bestowed with specialized components like proteins, intermediate filaments and other cytoskeleton components. Loricrin is one such protein that could play a role in the formation of a protective barrier as it is one of the late envelope proteins of the cornified cell envelope encoded by genes clustered in the so-called "epidermal differentiation complex" on human chromosome 1q21, [42,8]and this study was done to evaluate the expression of loricrin in hyperkeratosis, oral submucous fibrosis cases and normal mucosa specimens, as the epithelia subjected to stress show an increase in the relative amount of these proteins.

In conditions like OSF or hyperkeratosis, the chemical stimuli or the mechanical stress created due to areca nut chewing plus the calcium present in slaked lime can be the inciting factor in promoting the differentiation of the keratinocytes in non keratinising epithelia with the increased loricrin seen as a compensatory mechanism. It has been reported that loricrin is normally not expressed in non keratinising mucosa and other internal epithelia [55,56,40]and its expression in certain conditions could be a means of providing protection.

Sample standardisation

The samples collected were standardized for optimisation of the control sample with change in the concentration of the antibodies (1:200, 1:500) and the duration of the incubation period. Finally the procedures were standardized to a concentration of 1:500, and incubated for 1 hour at room temperature.

In our study it was found that the loricrin had a positive expression in all the three groups, (Group I Normal mucosal samples; Group-II hyperkeratosis and Group III OSF samples) with the normal mucosal samples showing 18.2% positivity, while hyperkeratosis samples showed 84.4% positivity and OSF samples showed a positivity of 66.7% and the positivity in all three groups was statistically significant ($p<0.001$). This is similar to the study done by Li *N et al* [49] where 63.6% of the OSF cases showed expression of loricrin, however the normal mucosal samples in their study did not show any staining.

Group-I Normal mucosal samples

Normal mucosal sample analysis in the study revealed that only one case had a habit history of chewing . Two samples showed mild positivity to loricrin in the stratum granulosum (SG) and could be attributed to the reason that biopsy had been taken distal to the retro molar region and might have been subjected to friction from the teeth. Katou *et al*[57] in his study when comparing expression of SPR2 (small proline rich proteins) and loricrin, found that loricrin was not expressed in the non keratinized mucosa or in transplanted skin without stratum corneum implying the altered presence of the marker as the possible adaptation of the keratinocytes to the changing environment of the oral cavity . Studies by Li N also showed negative staining among the normal mucosa[47, 49]

Group-II Hyperkeratosis

The hyperkeratosis group showed predominant number of cases (81.3%) having a habit history with chewing being the primary habit in 78.1%. Staining with loricrin

was positive in both the stratum granulosum(SG) and stratum corneum(SC), but showed no staining in the stratum spinosum. This was in accordance to the study by Katou *et al* where positive staining in both the layers were seen in transplanted skin and keratinized mucosa[57]

Majority of the cases stained positive in the stratum granulosum,with moderate staining being predominant among the staining intensities(43.8%).The stratum corneum showed both moderate and intense staining in about 6.2% of the cases.

We also analyzed the hyperkeratosis group by dividing it into those that could be caused by physical or chemical stresses based on the presence of epithelial dysplasia found in the histological sections. An increased number of cases in the chemically induced group (hyperkeratosis with epithelial dysplasia) had the habit of chewing (90.9%),while the physically induced group(hyperkeratosis without epithelial dysplasia) had (76.2%) of cases with habit history. The physically induced group showed loricrin positivity in about 90.47% ,while the chemically induced group showed positivity in 72.7% of the cases with both the cases staining predominantly in the stratum granulosum. This further compounds the fact that continuous irritation or micro trauma alters the normal signaling mechanisms present in the epidermis which are sensitive to the altered barrier /acute barrier disruption and also to varied external humidities. This has been contradicted by Selagae EM[58] *et al* in his study where the expression of loricrin is unchanged after barrier disruption, while a significant change has been observed with regard to involucrin another cornified envelope protein.

Areca nut is usually taken along with lime and the increased calcium concentration further inhibits the barrier recovery process. A simultaneous increase

in the lamellar body secretion rates are seen when calcium levels surrounding the stratum granulosum cells decline in parallel with the barrier disruption. [36]

Barrier disruption removes inhibitory lipids and allows influx of calcium and water movement into the SC interstices. SCCE, SCTE (kallikrein 7& 5 resp.) are serine proteases which regulate desquamation and are restricted by co-localised and co secreted protease inhibitors like SLPI(secretory leukocyte protease inhibitor) and SKALP(skin derived anti leukocyte protease) Both these proteases contain TGase substrate domains and are cross linked to loricrin residues in the CE and are minimally expressed in normal epidermis , but are increased in psoriasis and atopic conditions following wound healing or any barrier disruption.[55]

The presence of calcium in the mucosa of betel nut chewers from the calcium hydroxide (slaked lime) was confirmed by C R Trivedy *et al* [59]by doing a von kossa staining suggesting its presence both intra and intercellularly.

Group-III OSF

Oral submucous fibrosis is a chronic insidious disease with an obscure pathogenesis. Areca nut chewing is considered to be a key factor implicated in its pathology. In our study the habits were present in 93.3% of the cases in the OSF group indicating a strong causal association between the two.

While there was a 66.7% positivity in loricrin expression, further examination for staining among the different strata within the epithelium of the oral sub mucous fibrosis group revealed that the stratum spinosum showed a positive staining in the OSF group alone and not in the other two groups. This could be an finding where a

rearrangement of the cytoskeleton during cell division could lead to abnormalities in the cornified envelope which could be responsible for the molecular events that occur during the formation of dysplasia and transformation into malignancy.[8,60] and could be related to the decreased cell density in the atrophic epithelium .There is further evidence in the decrease of the retinoic acid receptors (RAR). The RARs actually suppresses the differentiation of the epithelium and suppresses the effect due to increased calcium induction and normally there is no expression of loricrin in the presence of retinoic acid.[60] Mild, moderate and intense staining were observed in the stratum spinosum.

The staining was moderate in majority of the cases which had staining in the stratum granulosum though mild and intense staining reactions were observed in 13.3% and 16.7% of the cases respectively. This is concurrent with the findings of Li N *et al* [47]The p-value which was less than 0.005 was found to be statistically significant when comparing the staining intensity evaluated in stratum granulosum among the three groups. Four cases had positive staining in their stratum corneum.

The evaluation of OSF by histological grading into early intermediate and advanced stages was done(data not included) and the expression was compared in all three stages, this revealed that an increased expression was seen in the early and intermediate stages with the advanced stage showing a decrease in the staining.

In our study,(data not included) the area of greatest concentration of the staining(nucleus or cytoplasm) was also observed which showed 14 cases with greater concentration of the stain in the nucleus ,OSF and physically induced hyperkeratosis(hyperkeratosis without dysplasia) samples showed 6 cases each

while chemically induced hyperkeratosis(with dysplasia) showed two samples exhibiting this feature.

Intra nuclear accumulation of the loricrin in the granular layer has been seen in some cases due to the introduction of potential nuclear targeting motifs in the Arginine rich sequences of the mutant c-terminal peptide that lack the normal protein. This alters the transglutaminase mediated cross linking of the peptide to other loricrin and other components of the CE, leading to changes in its flexibility and disruption of CE. [61]

Environmental stimuli can stimulate the epidermal keratinocyte and can induce the production of inflammatory cytokines and chemo tactic factors in an antigen independent fashion this can later lead to production of substances like interferon gamma and tumor necrosis alpha. This could also imply that there is a fair chance of the habit inducing mechanical stress leading to barrier function alteration.[34]

The directly activated keratinocytes can also influence endogenous or recruited immunocompetent cells. Within the groups the habit was predominantly seen in OSF with about 51.9% of them having the chewing habit. While 46.3% in the hyperkeratosis group and 1.9% among the normal mucosa cases had any history of habit. A significant increase in the chewing habit among the groups were seen. ($P<0.005$)

A strong expression of loricrin has been seen in the majority of the cases in the hyperkeratosis and OSF group in our study. The expression has been predominantly in the stratum granulosum layer. while a slightly reduced staining is seen in the stratum corneum which could be attributed to

- ❖ Reduced affinity of the antibodies to the epitopes due to continued cross linking
- ❖ Loss of epitope due to proteolysisthat occurs concomitantly with other differentiation products
- ❖ Masking of the epitope by other cellular components.[56]

A similar finding by Katou F *et al*[57] has been done where they found that Loricrin was strongly expressed in the SG of normal skin, transplanted skin retaining the SC and keratinized oral mucosa while its expression was absent in the transplanted skin lacking the SC and in the non-keratinized oral mucosa. Upregulation is also seen in orthokeratotic inflammatory skin diseases (e.g. lichen planus), with down regulation in parakeratotic inflammatory diseases (e.g. psoriasis vulgaris). The reason behind it being that Psoriasis is an hyper proliferative disease and the advanced terminal differentiaton products are diminished which includes loricrin along with keratins 1,10 and filaggrin.[56]

Previous *in vivo* experiments have suggested that the intrinsic properties of each type of epithelium are preserved after transplantation to other sites. Proved by cultured palate-derived epithelial cells transplanted to gingival mucosa expressed the differentiation programme typical of the original donor cultured sole derived epidermal cells transplanted to other body sites maintained their original epidermal phenotype. However, the transfer in both experiments was performed between basically similar environments, i.e. similarity in the transfer sites wet to wet and dry to dry surroundings, respectively. The findings of the present study may complement those *in vivo* experiments, in dealing with dry to wet conditions[57]. Keratinocytes might alter their properties only under completely different

conditions.Here the continuous micro trauma provided by the areca nut and other ingredients in chewing creates an environment similar to that of the dry regions and hence might be an adaptive response leading to its expression.

Retinoic acid affects the regulation of loricrin which has a similar expression to that of profilaggrin which is specific for different epidermal layers and follows expression of K1 and K10. The two methods of regulation could be either direct or indirect wherein the regulation of the gene expression is regulated or the differentiating process is regulated. Retinoic acid has also been seen to suppress the expression of loricrin *in vitro* and this can overcome the suppression due to calcium induction.[60]All these factors and more enlighten us as to the different mechanisms that could play a role in the transformation of the mucosa into something which has a potential for malignant transformation.

The present findings with regard to the expression of loricrin support the view on the adaptability of the epithelia to the new stimuli and suggest that it could be an important prognostic marker for an earlier diagnosis of a malignant transformation.

Summary and Conclusion

The aim of the study was to evaluate the expression of loricrin in oral submucous fibrosis hyperkeratosis and normal mucosa with the help of immunohistochemistry. The study comprised of a total of 73 archival tissue samples and were divided into three groups- ten normal mucosal samples,(Group-I);thirty two tissue samples of hyperkeratosis (Group-II) and thirty tissue samples of oral submucous fibrosis(Group-III).

- Normally expression of loricrin is limited to the stratum granulosum of the keratinising epithelia of the oral cavity and the epidermis. It is absent or not expressed in the non keratinising epithelia or the lining mucosa of the oral cavity.
- In the present study, Loricrin positivity was seen in groups II and III with mild staining in group-I
- The expression of loricrin among the three stratas of epithelium namely stratum spinosum, stratum granulosum and stratum corneum was evaluated with the following results- oral submucous fibrosis specimens showed positive staining in all three stratas, hyperkeratosis specimens showed a positive expression in both stratum granulosum and stratum corneum
- The presence of staining being taken up by the stratum spinosum of oral submucous fibrosis specimens alone could be related to three factors
    - Decreased cell density-as seen by the atrophic epithelium
    - Micro trauma related to areca nut chewing
    - Decrease in the number of (RAR) Retinoic acid receptors.
- Increased expression is seen in the early and intermediate grades of OSF than in the advanced stage indicating its expression being directly related to

the compensatory mechanism kicking in as a early response to the stress at the site.

- Expression is also seen in the hyperkeratosis group further convincing the fact that loricrin's expression facilitates a modest level of protection to the changing environment.
- Chewing habit is seen in almost all the patients establishing the role of areca nut chewing as a key etiological factor in the pathogenesis of OSF by-inducing stress (both mechanical and chemical).
- Expression of loricrin thus could be an early indicator of the various changes that could take place as a result of the continuous physical stimuli and could be a prognostic marker in oral potentially malignant disorders like OSF and leukoplakia.

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Annexures

ANNEXURE -I

From,

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai

The dissertation topic titled **" Expression of loricrin in hyperkeratosis, oral submucous fibrosis and normal mucosa – An Immunohistochemical study"** submitted by Dr.Nithya S has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 26th September 2011.

Dr.K.Ranganathan
Secretary,
Ragas, IRB

Dr.S.Ramachandran
Chairman,
Ragas, IRB

ANNEXURE-II

DISSERTATION PROTOCOL

Title of the proposed research project.

Expression of Loricrin in Oral Submucous Fibrosis, Frictional keratosis (Hyperkeratosis) and Normal mucosa

Name and designation of the principal investigator:

Dr. Nithya. S

II Year Postgraduate student

Department of Oral and Maxillofacial Pathology

Name of HOD and staff in-charge:

Dr. K. Ranganathan, M.D.S, M .S (OHIO) PhD

Dr. K. Umadevi, M.D.S

Dr. Elizabeth Joshua, M.D.S

Dr. T. Rooban, M.D.S

Department where the project is to be carried out:

Study will be conducted in Department of Oral & Maxillofacial Pathology, Ragas Dental College & Hospital

Duration of the project:

One year

Rationale:

Loricrin, a major protein of the cornified envelope, is one of the differentiation markers of terminally differentiated keratinocytes. Abundance of Loricrin in keratinizing epithelium, subjected to considerable mechanical stress has led to the assumption that expression of Loricrin is essential for the function of these tissues**.** The coarse fibres of areca nut and

the continuous friction from occluding teeth are the major causes of mechanical stress to the oral mucosa in oral submucous fibrosis and frictional keratosis (hyperkeratosis) respectively. Hence, the role of Loricrin in oral epithelium needs to be studied in these two lesions.

Hypothesis:

There is no difference in the expression of Loricrin in the epithelium of oral submucous fibrosis, hyperkeratosis and normal mucosa.

Aim:

To evaluate the expression of Loricrin in the epithelium of Oral submucous fibrosis , hyperkeratosis and normal mucosa.

Objectives:

- To study the expression of Loricrin in Paraffin Embedded tissue specimens of oral submucous fibrosis.
- To study the expression of Loricrin in Paraffin Embedded tissue specimens of frictional keratosis (hyperkeratosis).
- To study the expression of Loricrin in Paraffin Embedded tissue specimens of normal mucosa.
- To compare the expression of Loricrin in Paraffin Embedded tissue specimens of Oral submucous fibrosis, frictional keratosis (hyperkeratosis) and normal mucosa.

Signature of principal investigator

Signature of Head of Department

Remarks of committee:

Permission granted **YES** **NO**

Modifications / conditions

Materials and methods:

Sample:

Group I : 10 tissue samples of normal mucosa, to be obtained with patient's consent from the extraction site of 3rd molar

impaction.

Group II : 32 tissue samples of frictional keratosis (hyperkeratosis)

Group III : 30 tissue samples of oral Submucous fibrosis

Procedure:

Immunohistochemistry in archival samples, formalin fixed Paraffin Embedded Specimens.

Statistics to be used:

- Data analysis, to be done using SPSS (Statistical Package Of Social Science) version- 17.0
- The Intensity of staining with Loricrin between the groups (-,+,++,+++), could be analyzed using Chi-square test.

Equipments and chemical reagents needed:

Microtome

Light microscope

Autoclave

Hot air oven

Slide warmer

Coupling jars

Measuring jar

Weighing machine

APES coated slides

Slide carrier

Aluminium foil

Micro-pipettes

Toothed forceps

Electronic timer

Beakers

Rectangular steel tray with glass rods

Sterile gauze

Cover-slips

Reagents used:

1) Conc. HCl

2) Laxbro soln

3) APES (3 amino propyl triethoxy silane)

4) Acetone

5) Citrate buffer

6) Phosphate Buffered Saline (PBS)

7) H_2O_2 3%

8) Deionized distilled water

9) Nuclear Fast Red

10) Absolute alcohol

11) Xylene

Antibodies used:

1. Primary antibody – Loricrin Polyclonal Antibody
2. Secondary antibody – EXPOSE Rabbit specific –Horseradish Peroxidase
3. Chromogen – DAB-3, 3'- Diamino-Benzidine

Detailed budget plan:

Antibodies – Rs. 55,000

Laboratory Reagents – Rs. 3,000

Total – Rs.58,000

References:

1. Li N , Jian XC, Xu CJ et al, Expression of loricrin and cytochrome p450 3A5 in oral submucous fibrosis and their significance. Hua xi kou Qiang yi Xue Za Zhi 2009 Feb ; 27(1):29-33,
2. Ning Li, Xinchun Jian, Yanjia Hu, et al. Discovery of Novel Biomarkers in Oral Submucous Fibrosis by Microarray analysis. Cancer Epidemiology,Biomarkers & Prevention 2008 ;17:2249-2259.
3. Daniel Hohl ,Thomas Mehrel Ulrike Lichti, Maria L. Turner, Dennis R. Roop and Peter M.Steinert et al, Characterisation of Human Loricrin . The Journal Of Biological Chemistry 1991 Apr 266(5):6626-6636
4. Daniel Hohl ,Ulrike Lichti, Dirk Breitkreutz, Peter M. Steinert, and Dennis R Roop et al , Transcription of the Human Loricrin Gene In Vitro Is Induced by Calcium and Cell

Density and Suppressed by Retinoic Acid. Journal of The society for Investigative Dermatology,1990,sep 2794:535

5. Jackie R Bickenbach, Jeanette M. Greer, Donnie S. Bundman,Joseph A. Rothnagel, and Dennis R. Roop et al , Loricrin Expression Is Coordinated with Other Epidermal Proteins and the Appearance of Lipid Lamellar Granules in Development. Journal of The Society for Investigative Dermatology 1994 ,Dec 15.