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Immunohistochemical Expression of Toll-Like- Receptor-2 in Oral Epithelial Dysplasia Induced In Experimental Rats and Its Possible Relation to Mast Cell Count (An Animal Study)

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Abstract:*TLR-2 is a protein that is present in many cells, but its activation occurs due to microbial infection or genetic mutation. TLR-2 was significantly associated with the aggressive behavior of OSCC. Although the phenotypes of TLR-2 in different types of solid tumors have been extensively characterized, the expression and functional properties of TLR-2 in premalignant lesions remain to be determined. The infiltration of MCs has been shown to be an early and predictablecharacteristic of many pre-neoplastic cells, and may represent one of the earliest indications that a cell population has become committed to malignancy. This study was conducted to evaluated the expression of TLR-2 in OED and correlate it with MCC. A total of 48 adult male albino rats with an age range of 3 to 4 months and with a weight range of 100 to 200 gm were selected. The rats were randomly distributedinto two groups: control and experimental groups. Each group was further subdivided into two subgroups according to the time of sacrifice (after 6 and 9 weeks from the start of painting). Six rats were housed under the same conditions and did not receive any treatment. Forty two rats were anesthetized by ketamine 80-100 mg/kg intraperitonealy and xylazine 10-12.5 mg/kg (IP). The buccal mucosa was paintedwith a number 3 camel hairbrush. The rats had their buccal mucosa painted (topically) with DMBA and formaldehyde, 0.5% DMBA in acetone 3 days/week, and after 9 days 10% formaldehyde/water was used side by side with DMBA throughout the study period (6 and 9 weeks). Euthanasia of rats was done at 6 weeks (group A1andB1) and 9 weeks (group A2 and B2) interval from the start of painting The rats Euthanasia by an overdose of the anesthetic agents (1ml/100gm). The dissectedbuccal mucosa of rats were processed routinely to obtain 5µ thick sections. Thesesections were examined histologically by H&E stain and immunohistochemically byTLR-2, and TB stain, a special stain to detection the MCs. Moreover, the areapercentage of TLR-2 immunoexpression and the number of MCs in the differentintervals were measured by software Leica Qwin 500.The results showed a highly significant difference in both area percentage ofTLR-2 immunoexpression and mean MCC among the different experimental groups.After six weeks, the mean area percent of TLR-2 immunoexpression and themean number of MCs in low risk OED was significantly greater than that of controlgroup. Similarly, after nine weeks of the experiment, the mean area percent of TLR-2immunoexpression and the mean number of MCs in high risk OED was significantlygreater than that of control group. In addition there was a strong significantcorrelation between area percentage of TLR-2 and number of MCs among the studiedgroups.In conclusion, the immunoexpression of TLR-2 and MCC differ from low riskto high risk OED compared to normal buccal mucosa. TLR-2 and MCC could beused as early markers for malignant transformation*

Keywords*: epithelial dysplasia, premalignant lesions, precancer lesions, toll-like-receptors and mast cell.*

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I. Introduction

Oral epithelial dysplasia (OED) is a histopathological diagnosis that is associated with an increased risk of oral cancer. The role of tobacco and alcohol, as the two major risk factors, have been well documented **(Jaber, 2010).** Moreover, diet as well as tobacco and alcohol have been implicated in the large increase in oral leukoplakia which is the lesion classically associated with dysplastic changes. Of these, tobacco use and alcohol are identified as major risk factors, but interaction and/or summation of all factors may play a role **(Bánóczy et al., 2001).**Dysplasia is the step preceding the formation of squamous cell carcinoma (SCC) and is the first histopathological sign of cancer. Dysplastic changes are one of the signs of premalignant lesions which can be observed in histopathologic views especially in epithelial tissues. The risk of transformation to cancer is greater in dysplasia than in normal oral epithelium **(Kalra et al., 2012)** and **Anuradha et al., 2014).**Oral epithelial

dysplasia (OED), the histopathologic marker of a premalignant disorder of the mouth mucosa, may present clinically as leukoplakia, erythroplakia or leukoerythroplakia. Its presence in lesions of the oral mucosa is predictive by a variable rate (6.6% to 36%) for transformation into invasive SCC **(Shirani et al., 2014).**

Toll like receptors (TLRs) are a class of proteins that play a key role in both the innate as well as the adaptive immune systems. They are expressed by mast cells (MCs) , macrophages and dendritic cells **(Takeda and Akira, 2005).** The role of TLRs in cancer is controversial, as they can on one hand mediate signaling leading to inhibition of apoptosis and disrupt cell proliferation, and on the other hand, activate immunologic responses against cancer **(Mäkinen et al.,2015).**

Many TLRs (TLR1,2,4 and 6) showed an increased expression in oesophageal dysplasia. In oesophageal cancer high nuclear and cytoplasmic staining of TLR-4 was associated with metastatic diseases and poor prognosis. Little data is available as regards oral dysplasia **(Huhta et al.,2016).**

Moreover, many studies have revealed an increase in the number of MCs in oral dysplastic lesions and oral cancer compared to normal oral mucosa **(Mohtasham et al., 2010, Telagi et al., 2015, Veda and Vinay, 2015 and Ramsridhar and Narasimhan, 2016).** There is increasing evidence that MCs have a crucial function in tumorigenesis and tumor growth. TLRs are present on MCs as well as macrophages and dendritic cells **(Ng et al., 2011).** In this respect, this work aimed to evaluate the expression of TLR-2 in OED induced in experimental rats and to correlate this expression with mast cell count (MCC).

II. Material And Method

A total of 48 adult male albino rats, maintained in the animal house as an inbred colony (obtained from the Faculty of Medicine, Cairo University, Egypt) were used in this study. Rats with an age range of 3 to 4 months and t a weight range of 100 to 200 gms were selected for carrying out this experiment. The forty four rats of experimental group were anesthetized by intraperitoneal ketamine 80-100 mg/kg and xylazine 10-12.5 mg/kg according to The INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE guidelines (Application protocol 2012). The rats had their buccal mucosa painted (topically) with DMBA and formaldehyde; 0.5% DMBA in acetone 3 days/week and after 9 days10% formaldehyde/water was used side by side with DMBA throughout the study period using number.3 camel hair brush.

7,12-Dimethylbenz[a]anthracene (DMBA) was used to induce dysplasia in rats. 0.5 % DMBA was dissolved in100 µl acetone. Formaldehyde*/water (10%) was used after DMBA in induction of dysplasia, TLR-2**antibody (mouse anti-rat monoclonal primary antibody, dilution 1:100) is a biomarker for TLR-2 receptor expression in rat´s epithelium, Toluidine blue* stain specific for mast cell detection.

I-Housing of animals:

The animals were housed in a controlled environment (temperature 25 ± 2°c and 12 hr dark/light cycles). The animals were housed in a controlled environment (temperature 25 ± 2°c and 12 hr dark/light cycles).All rats were maintained on basic diet of regular rat chow and distilled water which is formulated to meet the nutrient needs of rodents throughout the study period. C- Rats were housed in stainless steel cages (four rats per cage) under the same conditions of temperature and humidity. Breeding mammals must have a solid floor at least in a nest box, bedding and additional nesting material.The rats were randomly distributed by Random Sequence Generator program (random.org).

Implementation of the allocation was done as follow:

Numbers from 1 to 48 were written on folded papers that were placed in opaque sealed envelopes, matching of threats with the numbers was done blindly through the technician in charge at the animal house. Each rat was attached to its number till the end, then the numbers were opened and threats were allocated in their groups according to the program's recommendations.

II-Grouping of rats :

Rats were divided equally into two groups as follows: Group A: Control group which contains 6 rats receiving no treatment and subdivided into two equal groups. Each group contains 3 rats according to the sacrifice date (after 6weeks (A1) and 9 weeks (A2)).Group B: Experimental group which contains 42 rats receiving DMBA and formaldehyde and subdivided into two equal groups. Each group contains 21 rats according to the sacrifice date {after 6weeks (B1) and 9 weeks (B2) from the start of painting}. (table1)

III- Euthanasia of rats:

Euthanasia of rats was done at 6 weeks (group A1and B1) and 9 weeks (group A2 and B2) interval from the start of painting; 21 rats for each interval(table 4) **(Kasem et al.,2014)**(table1)**.**

Table (1): Animal grouping and sacrifice dates:

	Control Group	Experimental Group
Number of rats	6	42
Induction of oral epithelial dysplasia	None	Using 0.5% DMBA in acetone. After 9 days 10% formaldehyde/water was used side by side with DMBA. Duration :Three times per week for 6 and 9 weeks from the start of painting
Sacrifice Dates	Group (A1): 6 weeks (3 rats) Group (A2): 9 weeks (3 rats)	Group (B1): 6 weeks (21 rats) Group (B2): 9 weeks (21 rats)

IV -Tissue processing and routine H &E staining technique

Buccal mucosa of rats was immediately dissected and fixed in 10% buffered formalin for 24 hours, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin.Sections of 5 microns thickness were cut from paraffin blocks and mounted on glass slides for routine H&E staining and subsequent examination under the ordinary light microscope to detect signs of dysplasia induced in rats' buccal mucosa.

V- immunostaining technique:

Paraffin blocks were cut into 4-5 microns thick sections and placed on positively charged (Opti-Plus) slides for immunostaining procedures. These slides provide better adhesion to the tissue sections and prevent them from detachment during staining **(Vara, 2005).**

Immunostaining for TLR-2 was performed using Ventana Bench mark autostainer (USA) as follows: deparaffinization and hydration of the tissue sections were done in descending grades of alcohol for 10 minutes each.

Staining for the formalin fixed tissue required boiling the tissue sections in l0mM citrate buffer, pH6.0 for 10-20 minutes followed by cooling at room temperature for 20 minutes (antigen retrieval step). The sections were then incubated in 0.3% hydrogen peroxide (H2O2) for 30Minutes to block the endogenous peroxidase activity.The sections were washed before the application of TLR-2 antibody at dilution of (1:100) under incubation temperature of 30 °C for 60 minutes, followed by application of the secondary antibody for 30 minutes. Diaminobenzidine (DAB) chromogen was applied to sections for 15 minutes at room temperature, Counter stain with Mayer's Hematoxylin was applied for 8 minutes, and Slides were extracted and arranged in racks. Slides were washed in tap water for 5 minutes and then dehydrated in ascending grades of alcohol for 5 minutes in each container. Slides-were cleared in xylene and then cover slips were applied.A TLR-2 positive reaction appeared as nuclear , cytoplasmic and membranous a brown stain, while pale brown stains or no stain were considered as negative immunoreactions **(Ng et al., 2011).**

Negative control:

In order to check for non-specific staining, as a negative control, one of the sections from each group was processed as the previously stated immunohistochemical steps, with replacement of the primary antibody by a negative control reagent (non-immune serum in PBS with 0.1% sodium azide). Fig. (10).

Positive control:

In order to ensure the effectiveness of this technique, tissue sections previously known to be positive for the antigen under examination, were stained. Positive staining was demonstrated by brown coloration. In case of the TLR-2, the positive control tissue was obtained from the kidneyFig. (11).

VII –Histochemical staining: technique (Toluidine blue –TB)

Four to five microns thick sections were cut and mounted on microscopic glass slides and subjected to TB histochemical staining for MC detection as follows IHC world, (2011): Sections were deparaffinized and hydrated in distilled water, sections were then stained in TB working solution for 2-3 minutes, tissue sections were then washed in distilled water for 3 changes.

, sections were dehydrated quickly through 2 changes of 95% and 100% alcohol (10 dips each) since stain fades quickly in alcohol. Sections were cleared in xylene; 2 changes for 3 minute each,sections were coverslipped with resinous mounting medium.TB stains MC granules with a violet/red purple stain in a blue background.

VIII - Assessment of histological sections:
The stained sections were assessed by two methods

1.Ordinary light microscopy:

Sections taken from animals sacrificed at 6 and 9 weeks interval were stained with H&E and studied under the light microscope to monitor the induced epithelial dysplasia. The examined cases were classified into two groups, based on dysplastic histopathological criteria; low risk group and high risk group. Mild dysplasia cases were considered as low risk group, while moderate and severe dysplasia cases as high risk group **(Kujan et al., 2006).**

2.Computer image analyser:

Area percent of TLR-2 immunoreaction was measured by the image analyzer computer system using the software Leica Qwin 500 (Germany) system,(fig. 1). The image analyzer was calibrated automatically to convert the measurement units (pixels) into actual micrometer units. The area percent for TLR-2 immunoreaction was measured in a measuring frame of 1920000 pixels.

Areas of the most intense brown staining were selected then the computer system converted the picture into a blue binary color that could be measured (fig. 2).
Three high power fields (x400) from each slide were measured. The mean area percent of TLR-2immunostaining was calculated for low and high risk dysplasia groups and was compared with the control group.

The number of TB stained MCs was counted. MCs in three high power fields (x400) for each slide were counted. The mean number of TB stained MCs was calculated for low and high risk dysplasia groups and was compared with the control group.

Fig. (1): A photograph of the image analyzer computer system.

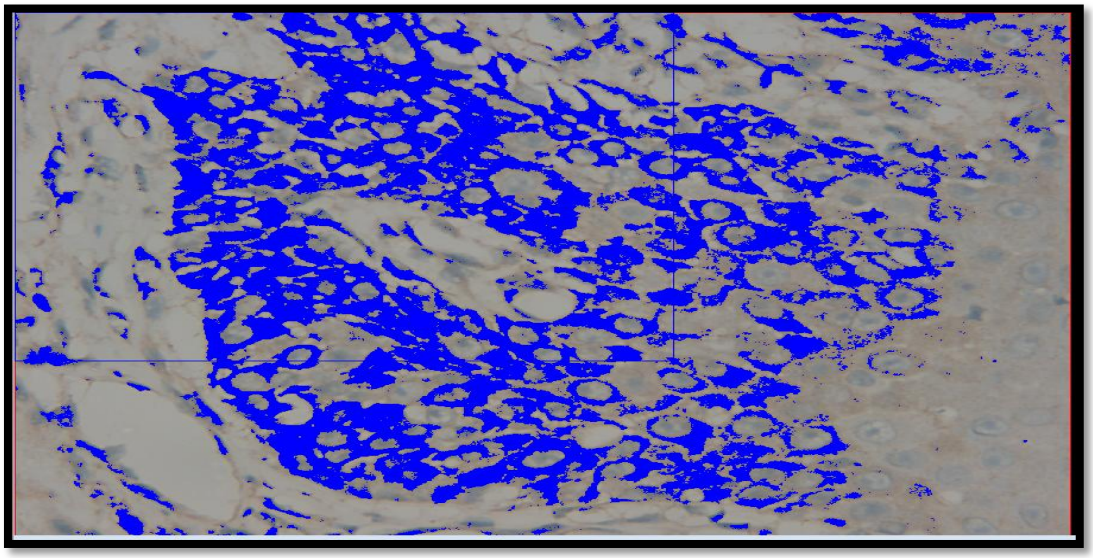


Fig .(2) :Copy of display of the computer image analyser system showing conversion of theimage into a blue binary colour to measure the area percent of TLR-2 immunostaining.

H&E findiding

A- Control groups (group A1 andA2):

Histopathological Results of H&E stained sections of control group (normal rats buccal mucosae) showed normal keratinized stratified squamous epithelium overlying normal connective tissue after both intervals 6 weeks and 9 weeks, fig.(3).

B-Experimental groups (group B1 and B2)

After 6 weeks from the start of drug painting, 20 cases showed mild dysplasia, 1 case showed hyperplasia and 1 case showed no obvious changes(fig.56,7.) After 9 weeks from the start of carcinogen painting, 12 cases showed moderate dysplasia, 7 cases showed severe dysplasia while 2 case showed mild dysplasia. Mild dysplasia cases and hyperplasia cases were considered in the low risk group(fig.4-7), while the moderate and severe dysplasia cases were considered in the high risk group(fig.8-9).

Histopathological findings of TLR-2 Imunostained Sections

Control groups (group A1 and group A2) Microscopic examination of TLR-2 immunostained sections of buccal mucosa of control group of both intervals (6 weeks and 9 weeks) showed negative or very faint immunoreactions (fig. 12).

Experimental groups (group B1and B2)

Group B1 (6 weeks following carcinogen painting (Low risk group)

Microscopic examination of TLR-2 immunostained sections of buccal mucosae in experimental rats (after 6weeks of carcinogen painting) (low risk group) showed positive immunoreactions (brown coloration) restricted to the basal 1/3 of the epithelium (figs.13-15). The reaction was noticed in the cytoplasm of basal or suprabasal of epithelial cells (figs. 13, 14 and 15). Few basal cells showed nuclearexpression (figs.14 and 15).In some cases an accentuated reaction was seen around the nuclear membrane in some cells (figs.13, 14 and 15). Inflammatory and endothelial cells showed positive immunoreactions (fig. 15).

Group B2 (9 weeks following DMBA and formaldehyde painting (High risk group)

Microscopic examination of TLR-2 immunostained sections of buccal mucosae in experimental rats (after 9 weeks of carcinogen painting) (high risk group) showed a positive immunoreaction (brown coloration) comprising almost all epithelia except the keratin layer (figs. 16, 17 and19) showing a more obvious reaction in the basal layers (fig.18). The reaction was mainly cytoplasmic (figs 16, 17and19). Expression at cell membrane was also seen in many prickle cells (figs17 and 18). In some cases an accentuated nuclear membrane reaction was seen the in some cells (figs.18, 19). Few basal cells showed a nuclear reaction (fig.19). Inflammatory cells showed positive

III. Histopathological Finding Of Toluidine Blue Stained Sections

The studied groups showed variable numbers of mast cells that appeared aslarge rounded cells infiltrating in the connective tissue. Red- purple cytoplasmic granules were obvious in some of them. In control group A1 and A2, MCs were scarcely detected in the connective tissue (fig.20). In the experimental groups B1 (6weeks following drug painting) and B2 (9weeks following drug painting), low and high risk groups respectively, MCs were demonstrated with variable degrees of inflammation, where the number of cells appeared relatively higher in high risk group which showed numerous MCs (fig.27) compared to low risk group (fig.21).

Statistical analysis:

Data from the two groups was collected, tabulated statistically analyzed and illustrated in tables and figures. The data was summarized as means and standard deviations. Collected data were analyzed using a SPSS statistical package.* Student t-test was performed to compare between each of low and high risk dysplasia groups with the control group and between low and high risk dysplasia groups. Results were considered significant at $p<0.05$.Pearson correlation test was used to correlate mean area percent of TLR-2 immunoexpression and mean MCC in the control`, low and high risk groups.

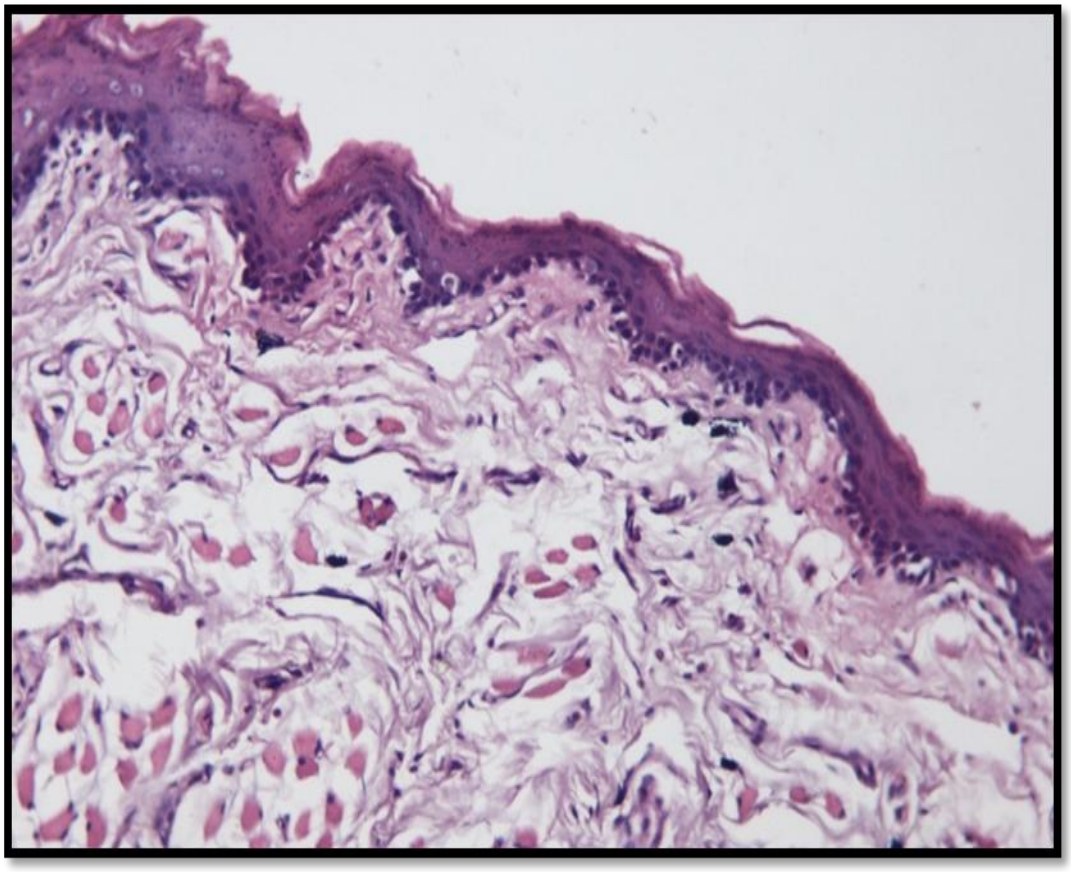


Fig.(3):Photomicrograph of buccal mucosa of a rat in the control group revealing normal keratinized stratified squamous epithelium overlying normal connective tissue (H&E, x200).

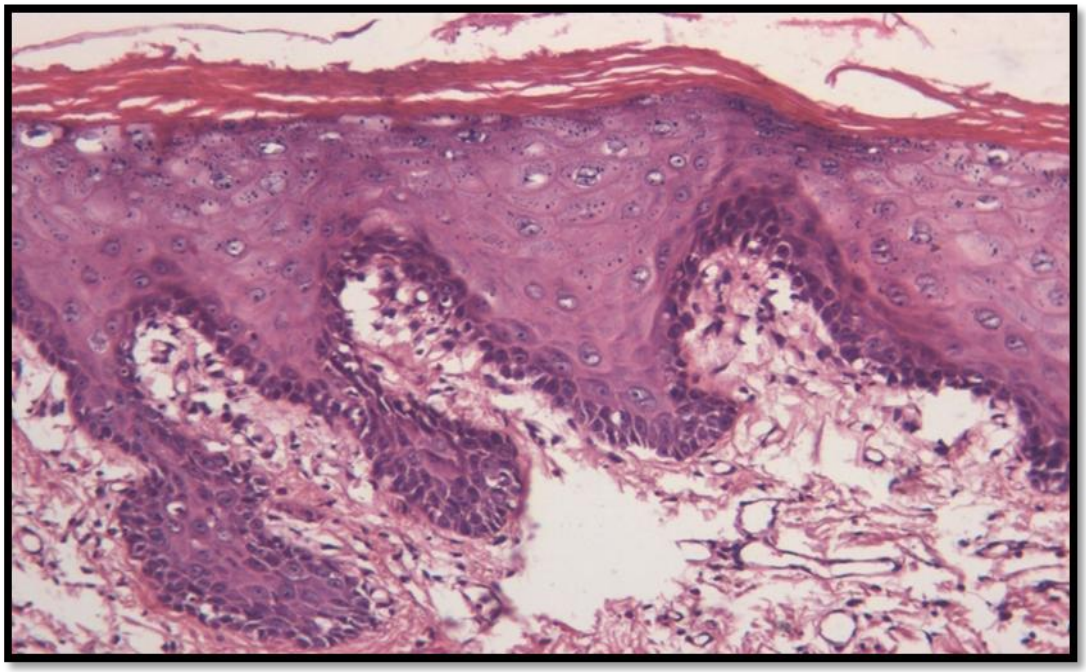


Fig. (4): Photomicrograph of low risk group (B1) showing excessive surface hyperkeratosis (black arrow),acanthosis of prickle cell layer and basilar hyperplasia .Notice the tear-drop shaped rete ridges (H & E X 200).

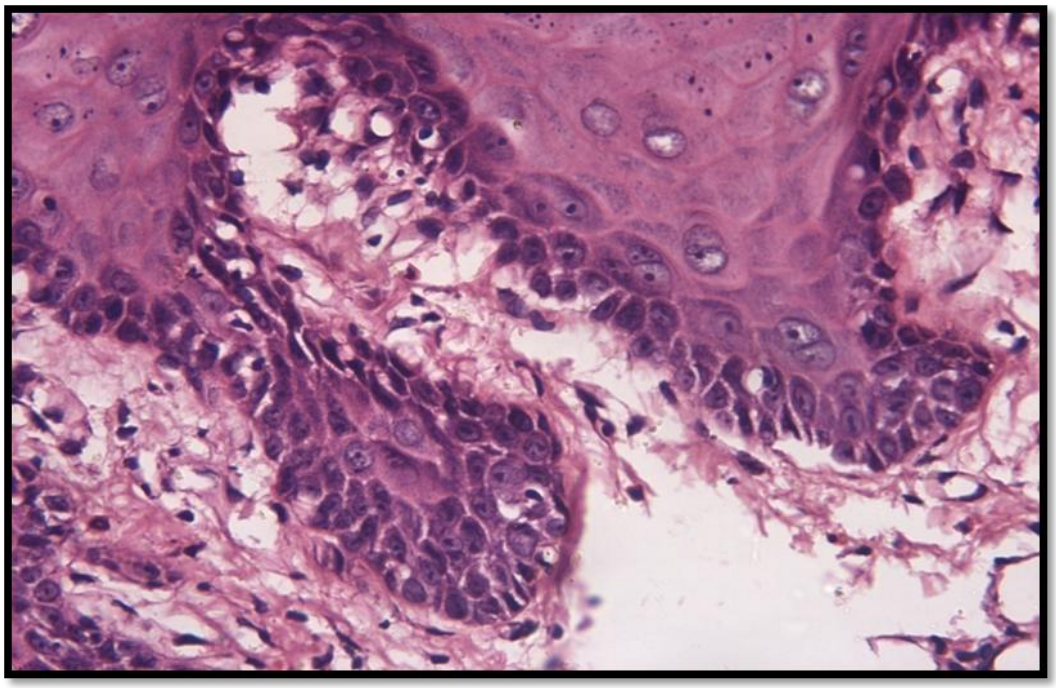


Fig. (5): High magnification of the previous photomicrograph showing loss of polarity of basal cell layer (white arrow),karyomegaly and hyperchromatism(yellow arrow), nuclear pleomorphism is also seen (green arrow) (H & E X 400).

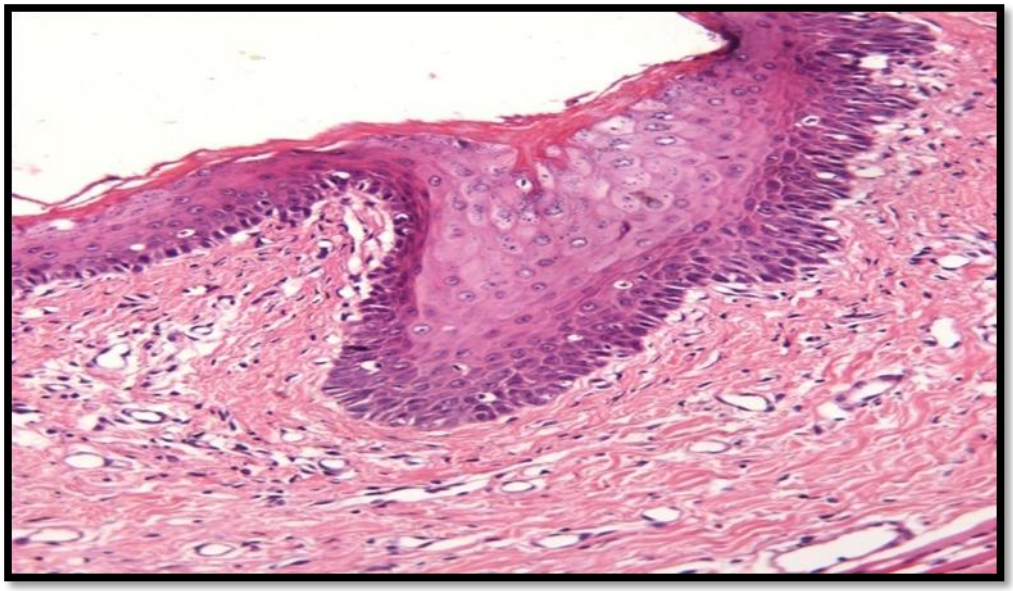


Fig. (6): Photomicrograph of low risk group (B1) showing surface hyperkeratosis. acanthosis of prickle cell layer, and basilar hyperplasia.The connective tissue shows few inflammatory cells (H & E X 200).

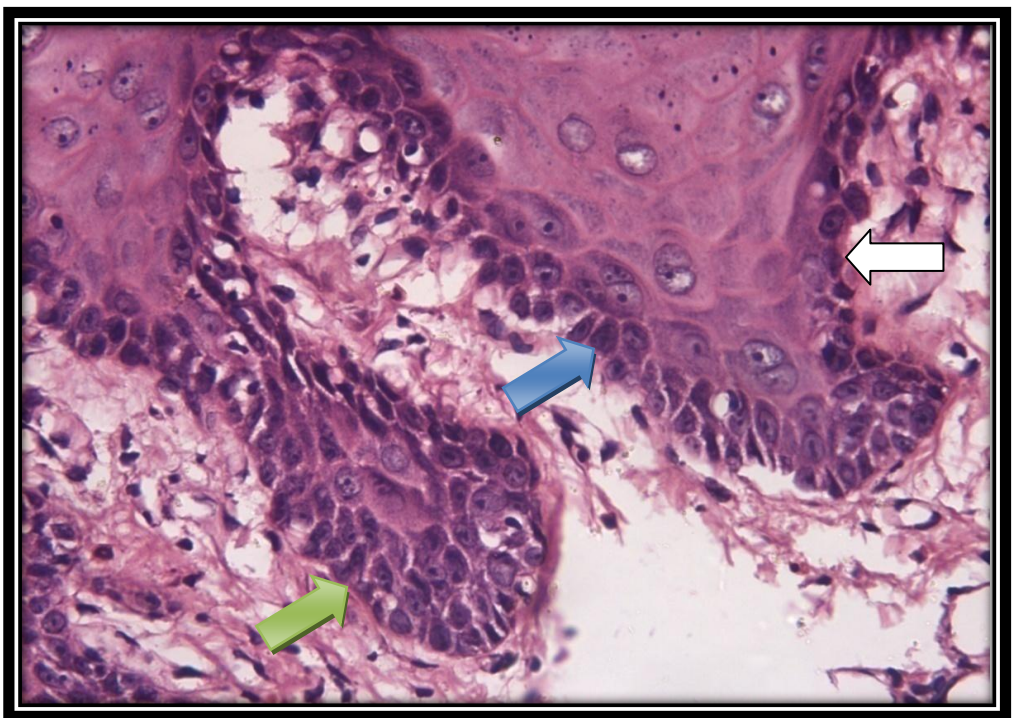


Fig. (7):Higher magnification of the previous photomicrograph showing loss of polarity of basal cell layer (white arrow), karyomegaly and hyperchromatism (blue arrow),as well as nuclear pleomorphism is also seen (green arrow) (H&EX 400).

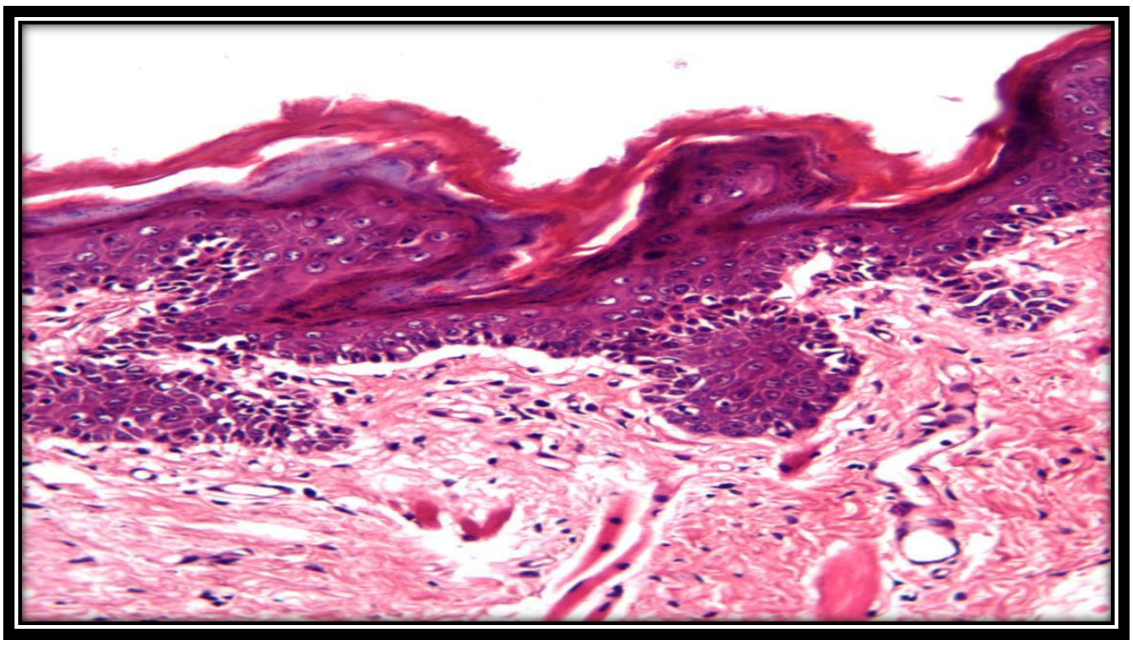


Fig. (8): Photomicrograph of high risk group (B2) showing excessive surface hyperkeratosis and surface papillary projections. Nuclear hyperchromatism, pleomorhpism and karyomegaly exceed the lower half of the epithelial thickness (H&EX 200).

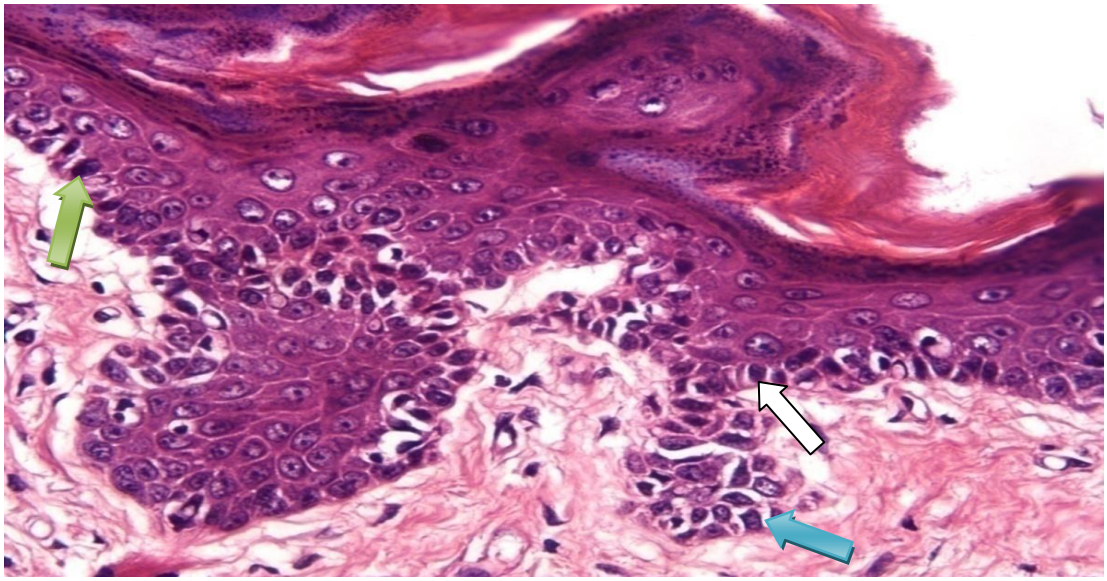


Fig. (9): Higher magnification of the previous photomicrograph showing nuclear hyperchromatism (white arrow), pleomorhpism (blue arrow), and karyomegaly (green arrow). Loss of basal cell polarity is obvious in the club shaped rete ridegs (H&EX 400).

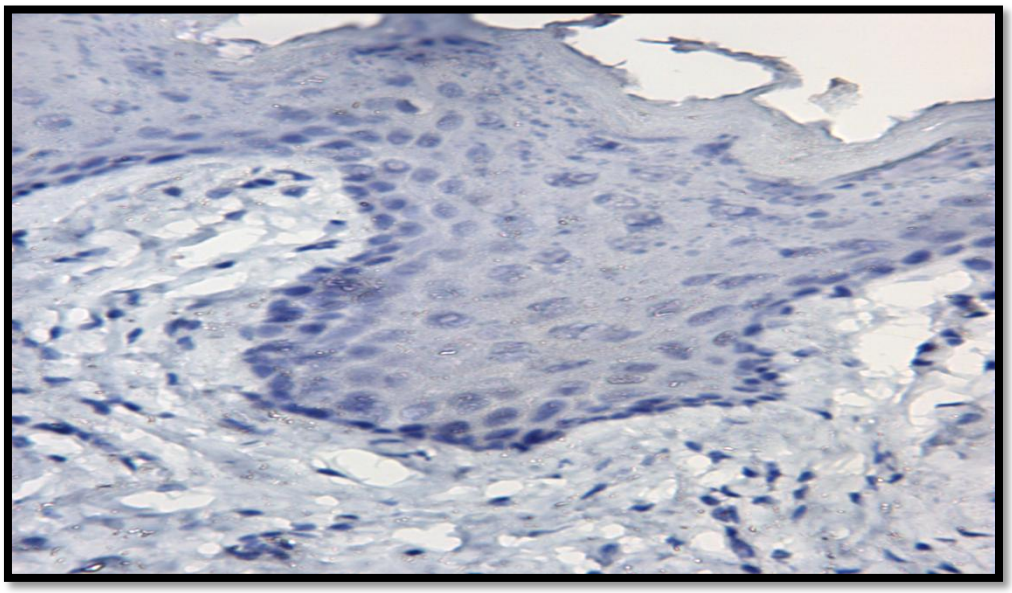


Fig.(10) Photographic showing negative control (TLR-2 x200).

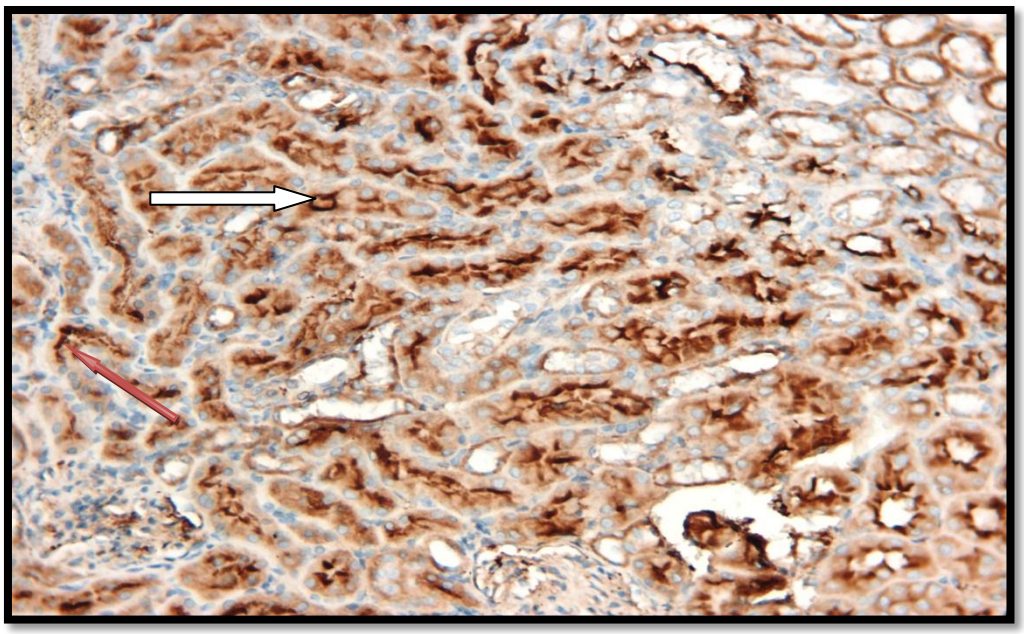


Fig.(11) Photomicrograph of positive control section showing cytoplasmic (white arrow) and nuclear (red arrow) TLR-2 immunoexpression in renal cells of kidney (TLR-2 X 200).

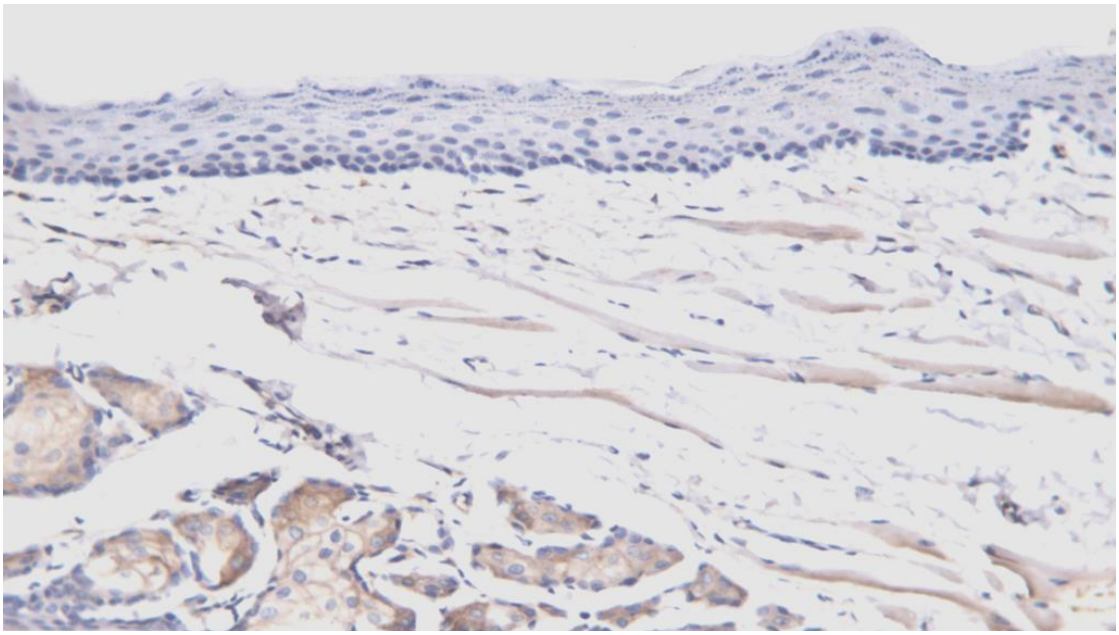


Fig.(12) Photomicrograph showing faint TLR-2 immunoexpression in normal stratifiedsquamous epithelium of the control group (TLR-2x200).

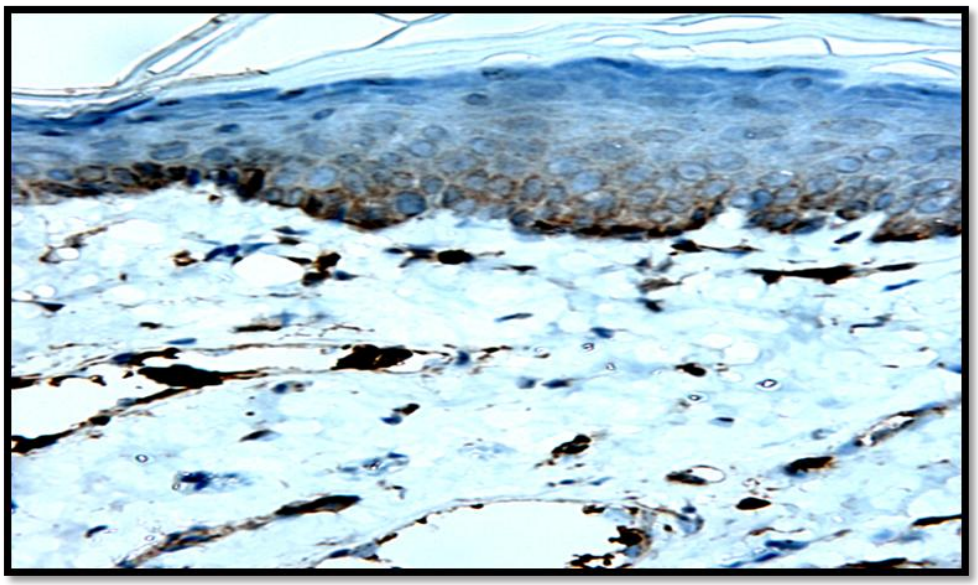


Fig.(13): photomicrograph showing cytoplasmic TLR-2 immunoexpression (TLR-2x200) .

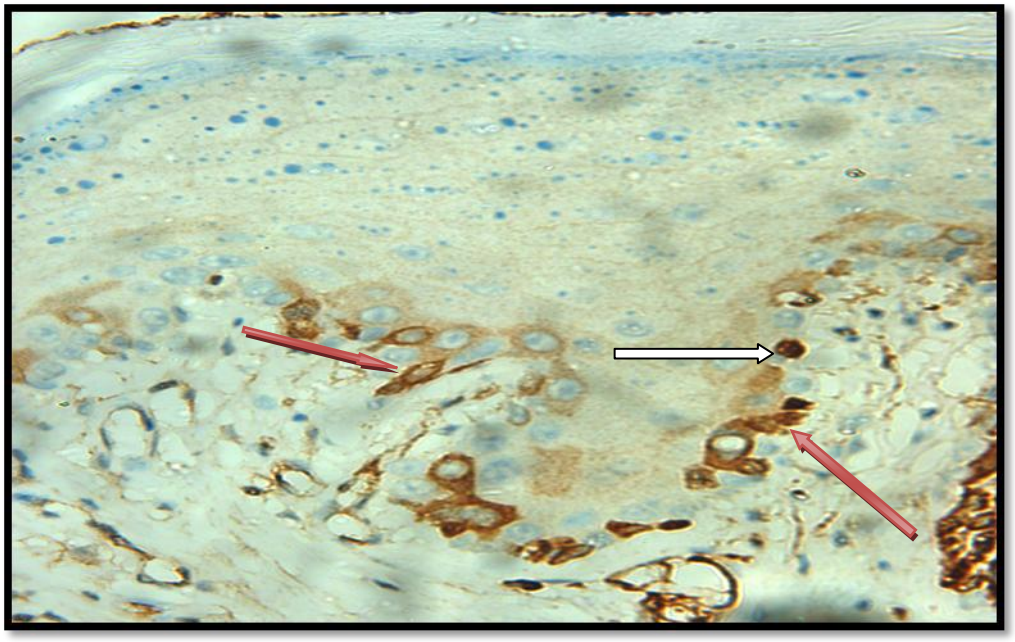


Fig.(14) : Higher magnification of the previous photomicrograph showing cytoplasmic TLR-2 immunoexpression in basal and suprabasal epithelial cells(red arrows). Few nucli showed a postive immunoreactions (white arrow) (TLR-2 X400).

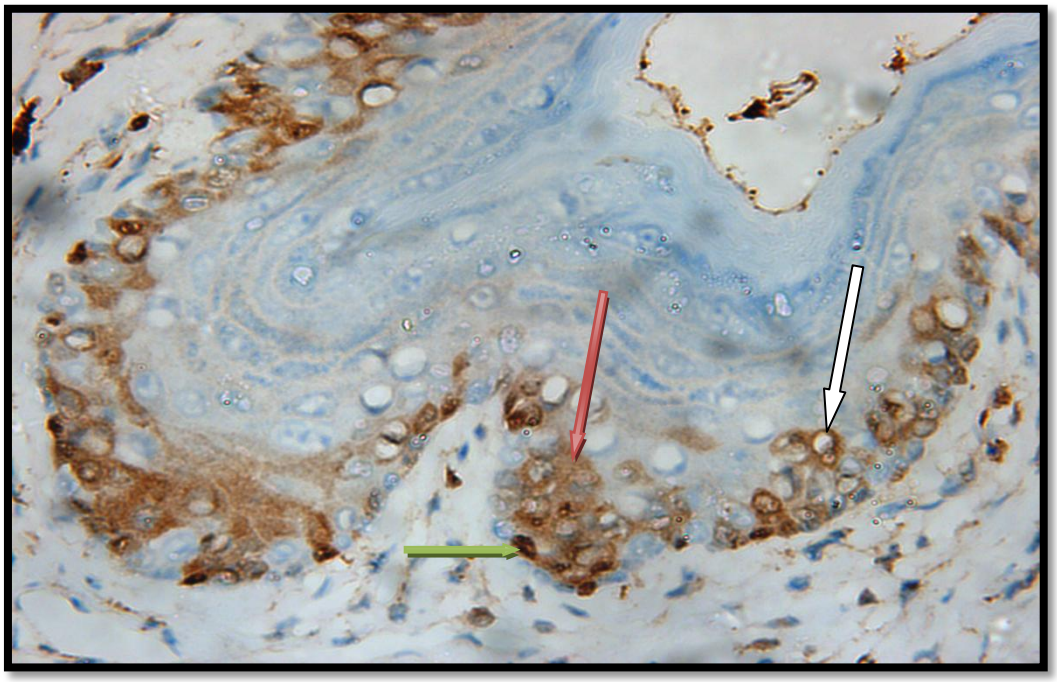


Fig. (15): photomicrograph showing diffuse and homogeneous cytoplasmic TLR-2 immunoexpression in most basal and suprabasal cells (red arrow). Nuclear expression is noticed in some basal cells (green arrows). An accentuated nuclear membrane reaction is seen around the in some cells (white arrows) (TLR-2 X 400).

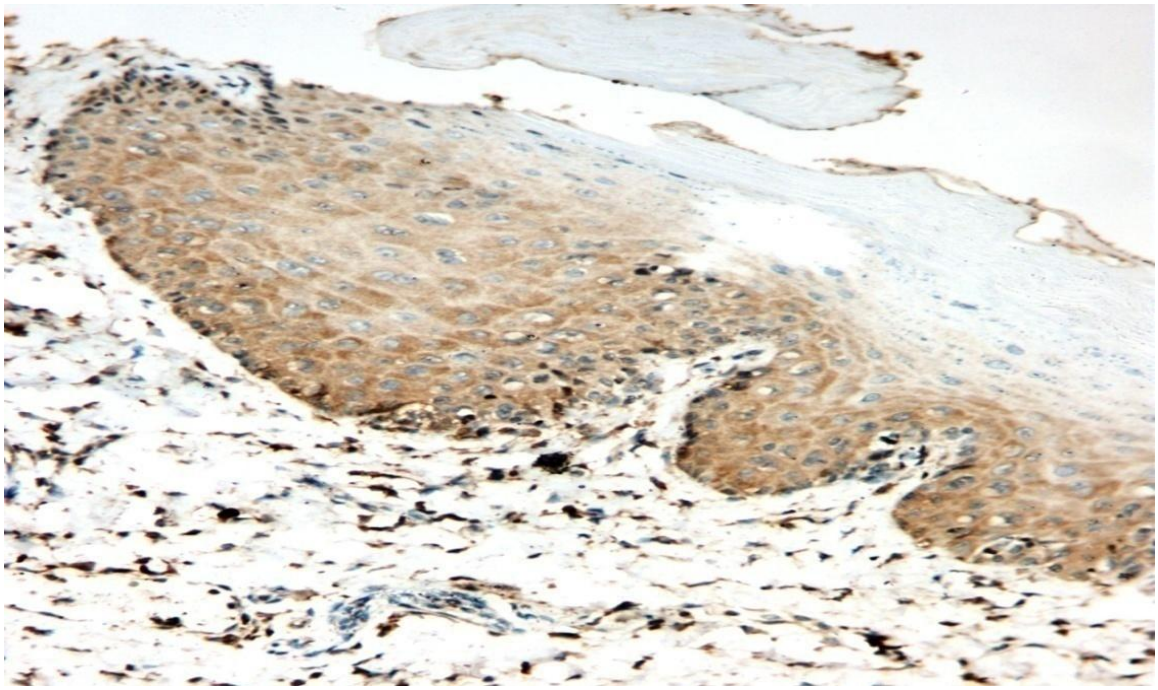


Fig.(16): Photomicrograph of group B2 (high risk group) showing TLR-2 immunoreaction in the lower two thirds of the epithelium, (TLR-2 X 200).

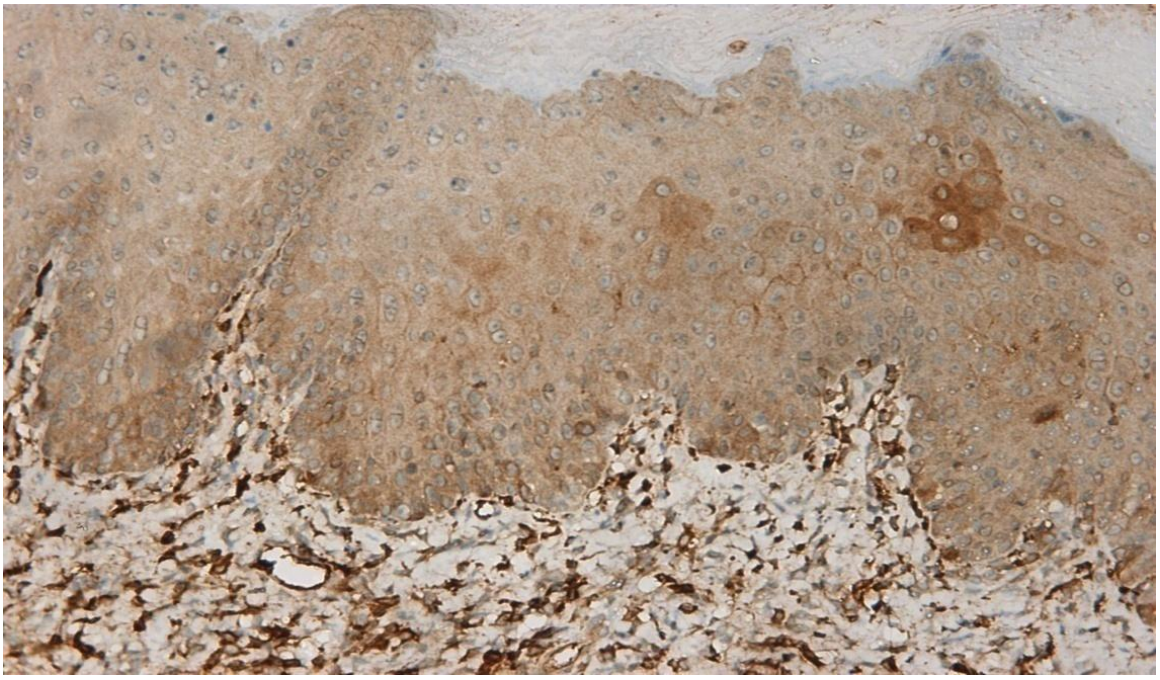


Fig. (17): Photomicrograph of group B2 (high risk group) showing a homogenous and diffuse cytoplasmic TLR-2 immunoexpression in all epithelial layers showing a more obvious expression in few localized areas (red arrows) (TLR-2 X 200).

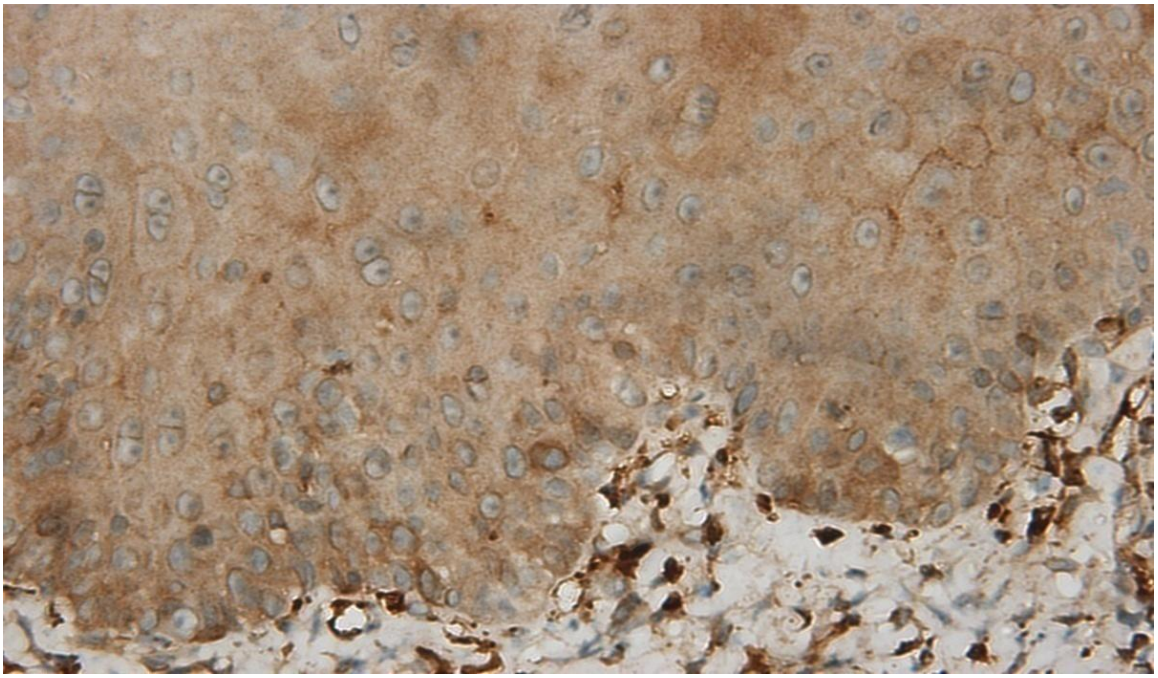


Fig.(18): photomicrograph showing a membranous reaction (red arrows) in some prickle cells. An accentuated reaction is noticed around the nuclear membrane in some cells (yellow arrows) (TLR-2 X 400).

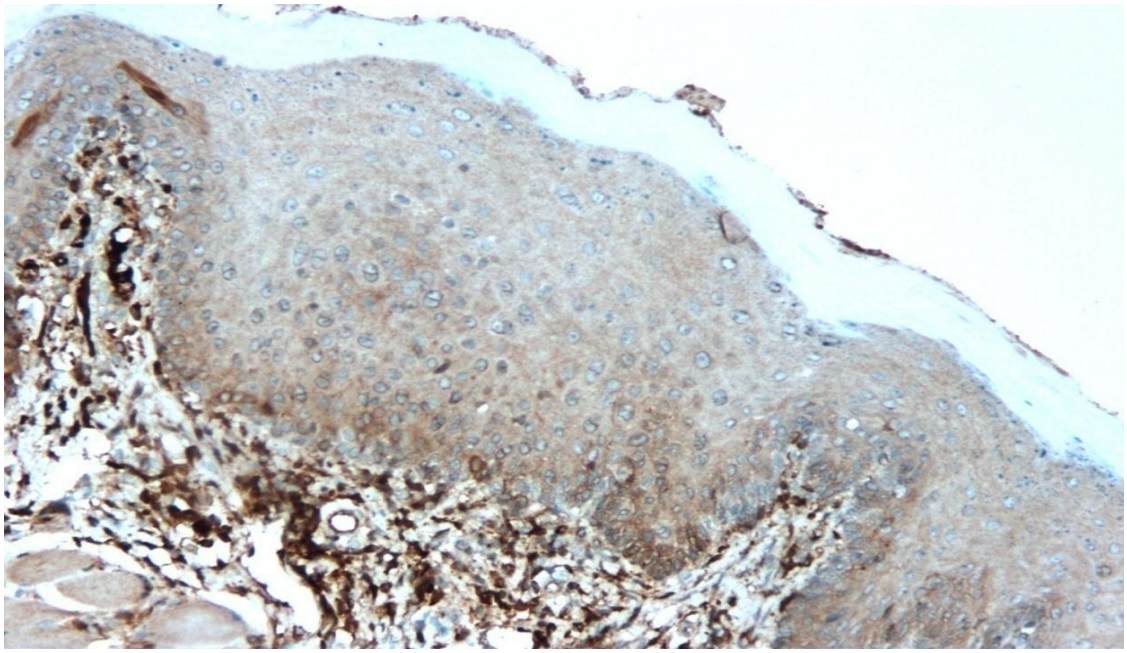
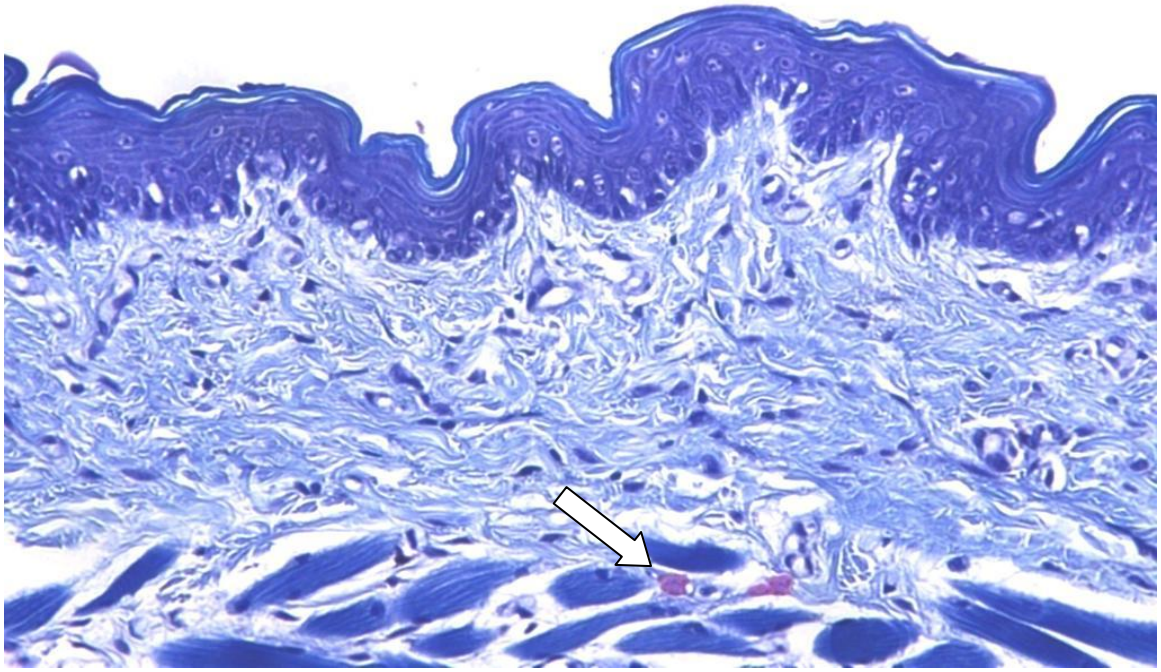


Fig. (19): Photomicrograph of group B2 (high risk) showing TLR-2 immunoexpression in all layers of the epithelium except in the keratin layer with more obvious reaction in the basal layers. Few basal cells showed nuclear TLR-2 immunoexpression (red arrow) (TLR-2 X 200).



Figs.(20) : Photomicrograph of normal buccal mucosa of control group(arrow) (TBx200).

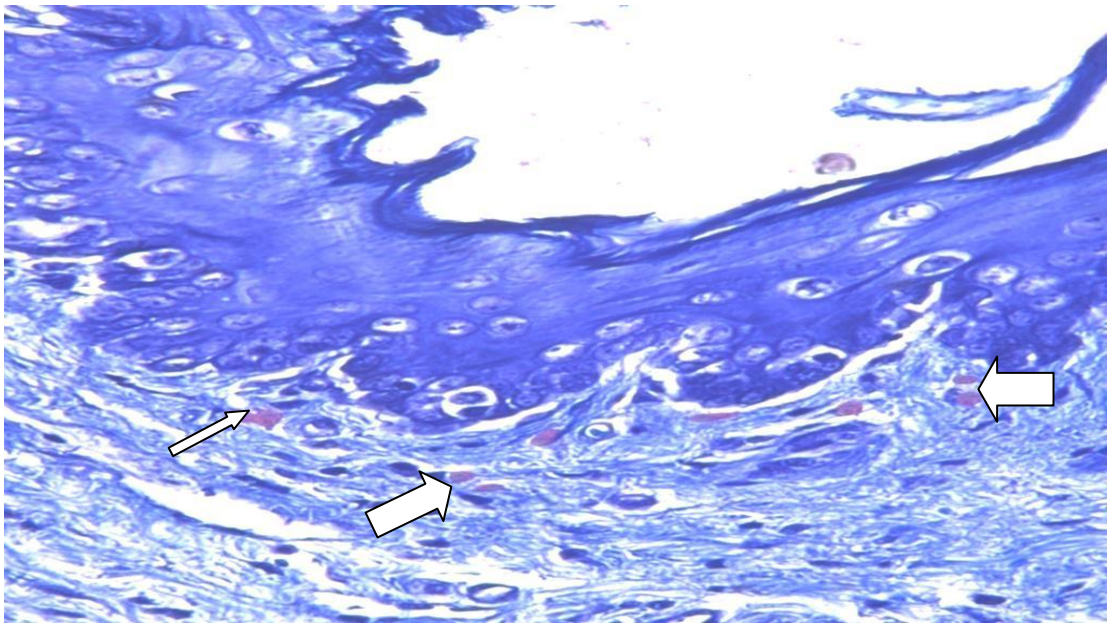


Fig. (21): photomicrograph of low risk dysplasia showing few rounded largeMCs with cytoplasmic red granules (arrows) haphazardly scattered in the subepithelial connective tissue (TB X400).

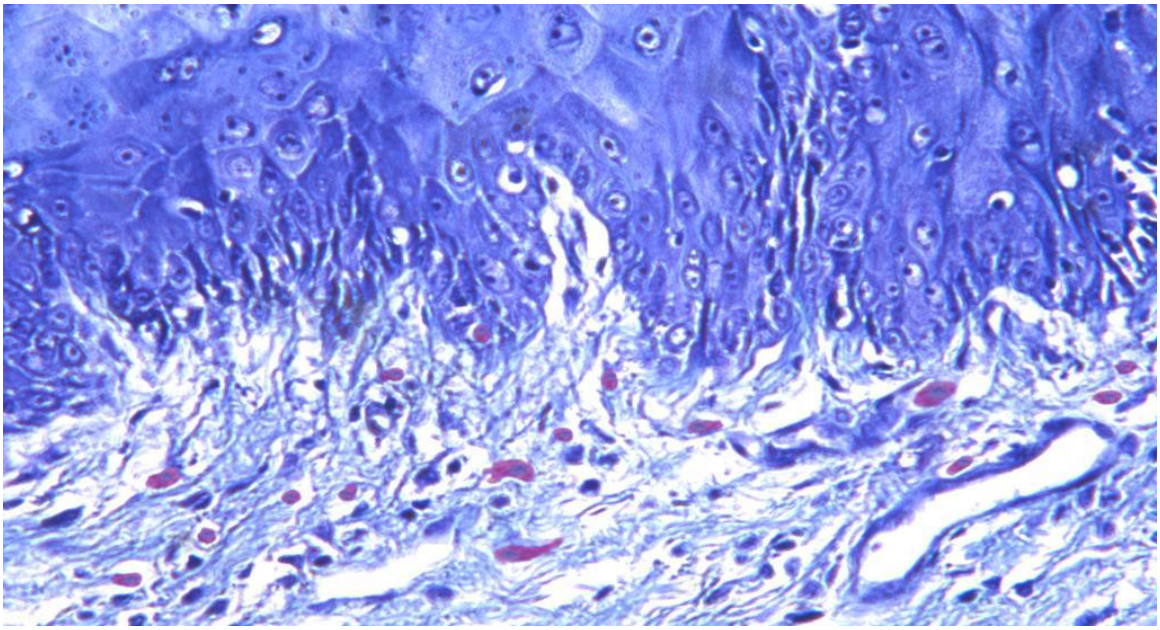


Fig. (22): photomicrograph of high risk dysplasia showing many rounded and spindle shape MCs (arrows) with cytoplasmic red purple granules diffusely scattered in the connective tissue just underneath the dysplastic epithelium (TB X400).

Table(2) : Mean area percentage values of control group (group A1) and low risk group (group B1) (Student T-test).

	Group	**Mean**	**SD**	**P value**
Area percentage	**control**	**5.85**	**1.94**	**<0.0001***
	low risk	**28.85**	**2.73**	

SD= standard deviation
P value <0.05 are statistically significant

Table (3): Mean area percentage values of control group (group A2) and high risk group (group B2) (Student-t-test)

	Group	Mean	SD	P value
Area percentage	control	5.85	1.94	<0.0001*
	High risk	88.6	3.72	

SD= standard deviation
P value <0.05 are statistically significant

Table (4): Mean area percentage values of low risk group (group B1) and high risk group (group B2) (Student-t-test)

	group	Mean	SD	P value
Area percentage	Low risk	28.85	2.73	<0.0001*
	high risk	88.6	3.72	

SD= standard deviation
P value <0.05 are statistically significant

Table (5): Mean MCC in control group (group A1) and low risk groups (groupB1) Student-T-test

	Group	Mean	SD	P value
MCC	**control**	**4.33**	**0.51**	**<0.0001***
	low risk	**13.5**	**1.87**	

SD= standard deviation
P value <0.05 show statistically significant

Table (6): Mean MCC in control group (group A2) and high risk group (group B2) (Student- t-test)

	Group	Mean	SD	P value
MCC	**control**	**4.33**	**0.51**	**<0.0001***
	High risk	**33.33**	**3.55**	

SD= standard deviation ,P value <0.05 are statistically significant

Table (7): Mean MCC in low risk group (group B1) and high risk group (group B2) (Student t- test)

	Group	Mean	SD	P value
MCC	Low risk	13.5	1.87	<0.0001*
	high risk	33.33	3.55	

SD= standard deviation
P value <0.05 are statistically significant

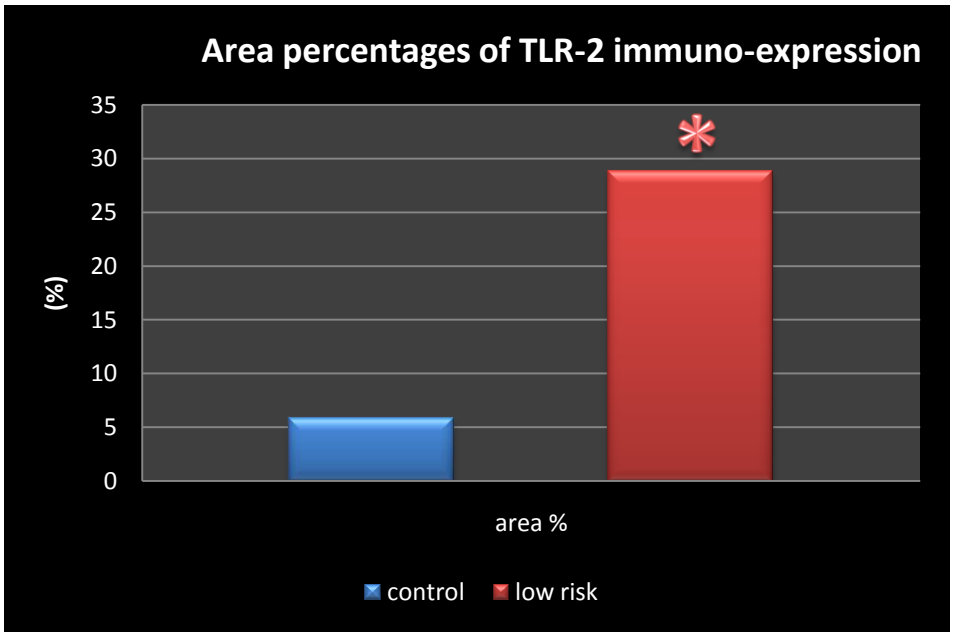


Fig. (23): Bar chart showing mean area percentage values of control group (group A1) and low risk group (group B1).

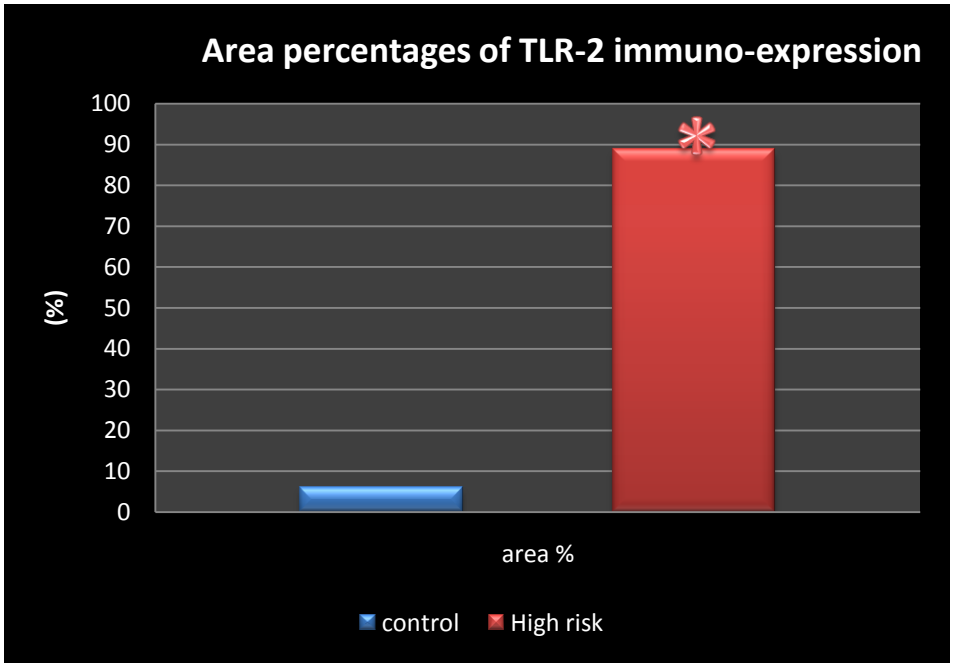


Fig. (24): Bar chart showing mean area percentage values of control group (group A2) and high risk group (group B2).

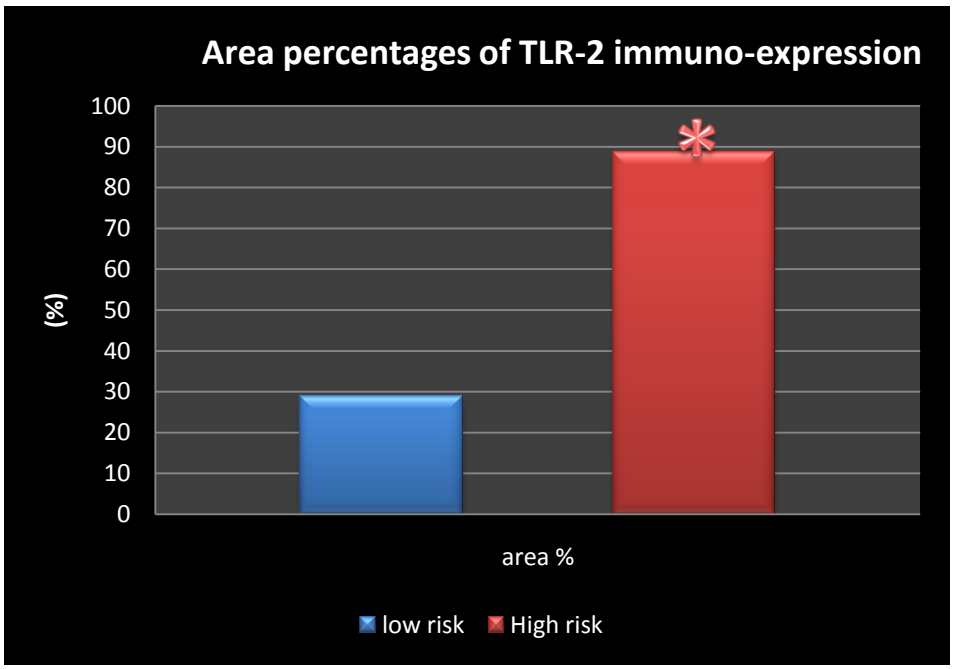


Fig. (25): Barchart showing: mean area percentage values of low risk group (group B1) and high risk group (group B2).

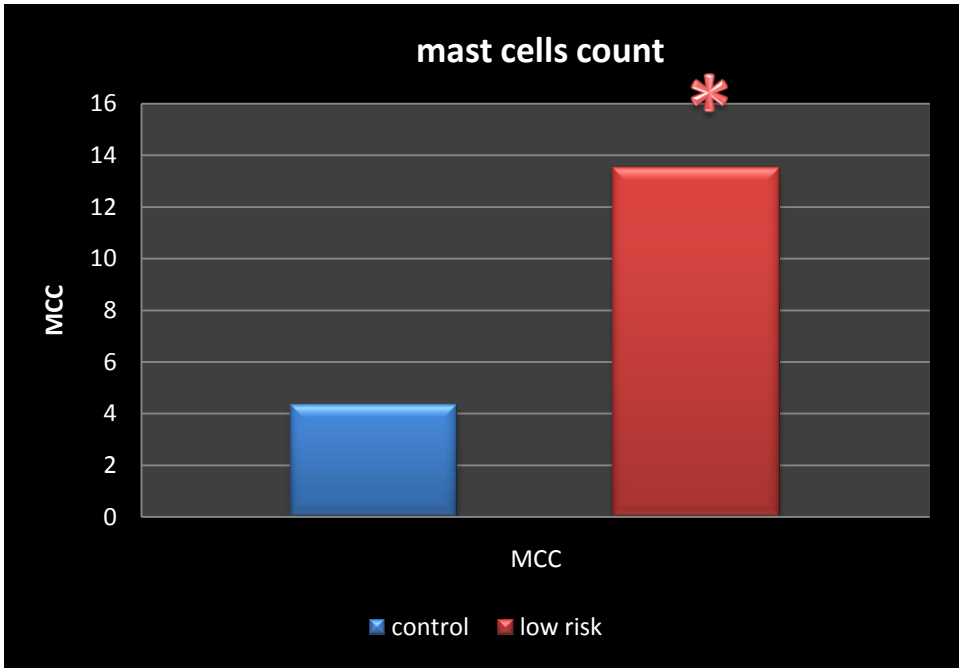


Fig. (26): Bar chart showing mean MCC in control group (group A1) and low risk group (group (B1).

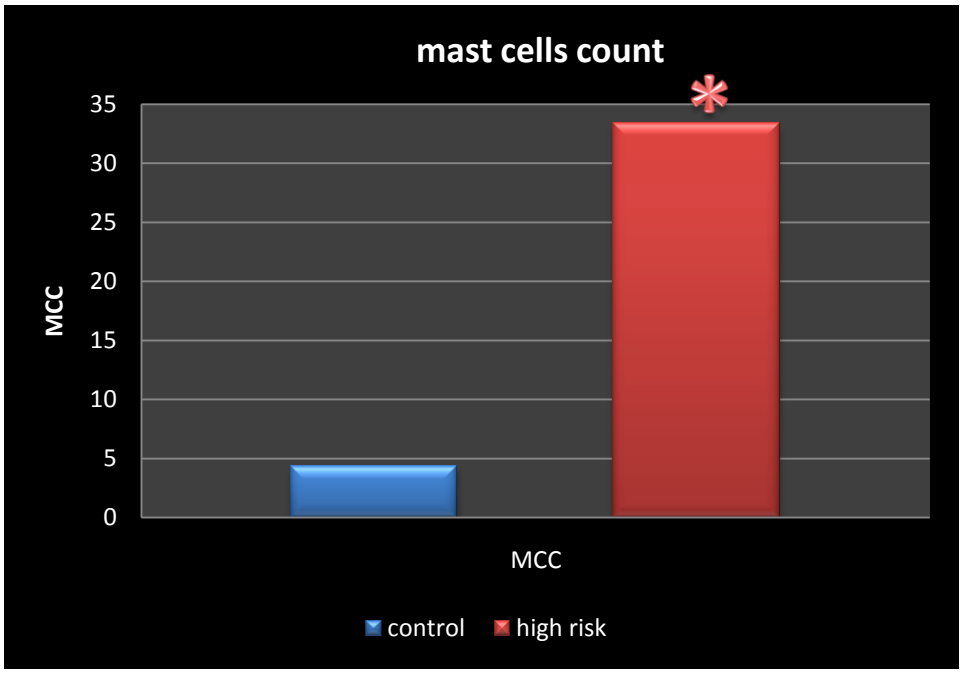


Fig. (27): Bar chart showing means MCC in control group (group A1) and high risk group (group B2).

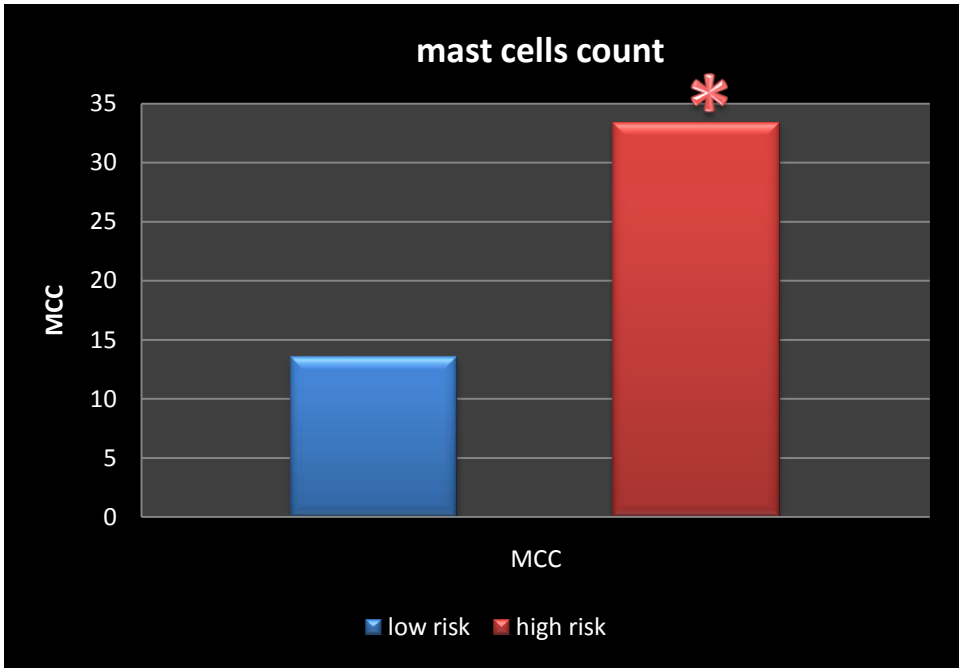


Fig. (28): Barchart showing mean MCC of low risk group (group B1) and high risk group (group B2).

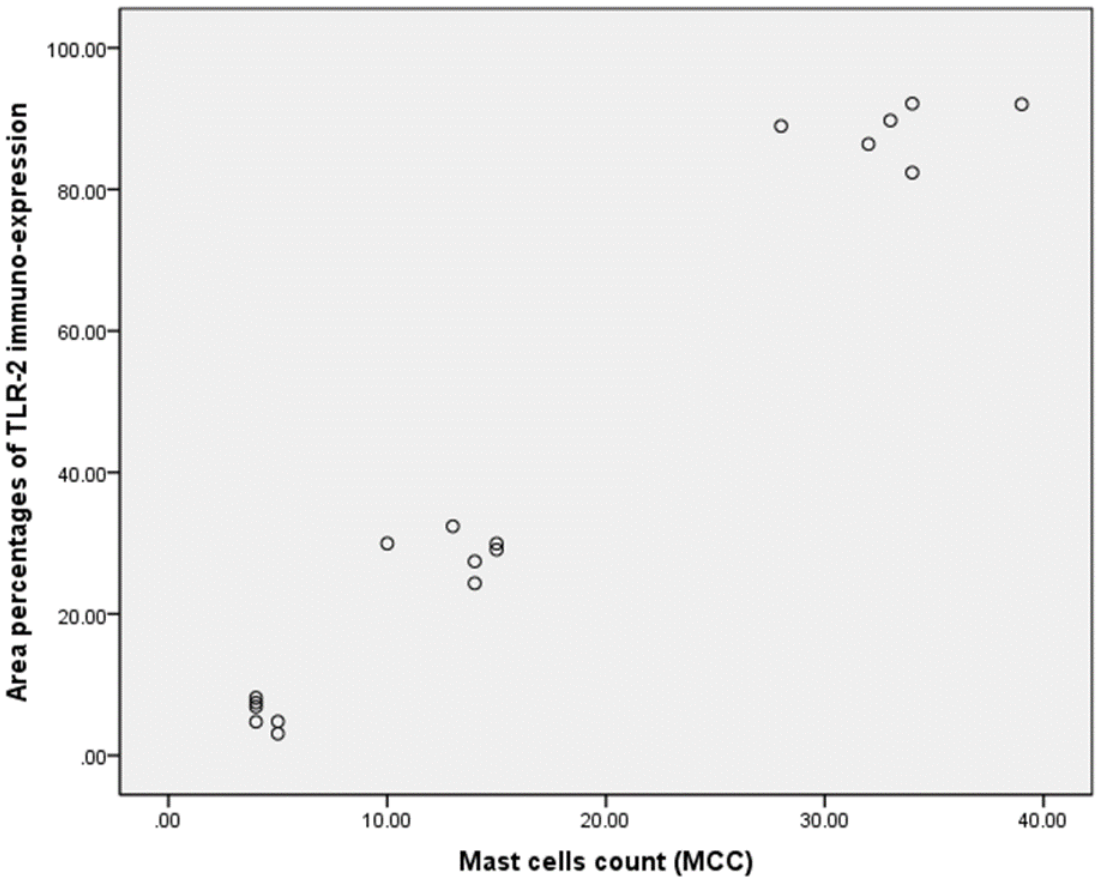


Fig. (29): Correlation between area percentages of TLR-2 immuno-expression and MCC among the low and high risk groups

IV. Discussion

Animal models which recapitulate the human condition are critical to furtherunderstanding of the molecular, biological and clinical aspects of many diseases including cancer. The hamster buccal pouch and the rat models of oral carcinogenesisare well known surrogate models for the human condition**(Tang et al., 2004).** Ratmodels enable the development and testing of new approaches in prevention,treatment, identification of early diagnostic markers and an understanding of thegenetics and biology of tumor initiation and progression in an animal model whosegenome is most similar to humans **(Twigger et al., 2007).**

TLRs are intracellular transmembrane proteins that are expressed on the cells of the immune system such as MCs, macrophages and dendritic cells. In addition, they are present on non-immune cells such as keratinocytes of skin, oral mucosa, gastrointestinal and female reproductive tract lining. On these lining epithelia, TLRs act as sensors where they recognize pathogens and are activated when the epithelium is disturbed **(Rich et al .,2014).**

TLR-2 has a significant role in oral cancer and precancerous lesions as expressed in HNSCC. Activation of TLR-2 promotes tumor growth through activation of NF-κB and MAPK pathways **(Farnebo et al. 2014).**

The MC is a granular white blood cell. It contains large cytoplasmic granules which stores a variety of mediators as histamine and heparin (Da Silva et al., 2014). An increase in MCC has been seen in many oral dysplastic **lesions (Mohtasham etal., 2010, Telagi et al., 2015 ,Veda and Vinay, 2015 and Ramsridhar and Narasimhan, 2016)** suggesting a role played by MCs in oral dysplasia.

The present study attempted to evaluate the expression of TLR-2 in oral dysplastic epithelium and correlate this expression with MCC in low and high OED groups in comparison with normal mucosa.

This work was carried out on forty eight rats to detected immunoexpression of TLR-2 after induction of OED for two intervals (6 and 9 weeks) using chemical carcinogens DMBA and **formaldehyde (Kasem et al., 2014).**

Area percent of TLR-2 immunorexpression and MCC were measured by usingthe image analyzer computer system which is an accurate and reproducible methodand avoids human subjectivity **(Decaestecker et al., 2009).**

Results of the present work illustrated negative or faint TLR-2 immunoexpression in normal epithelium of buccal mucosa of the control group. This finding is consistent with **Ng et al.(2011)** who declared negative TLR-2 immunostaining in oral keratinocytes of hyperplastic epithelium. This finding could be explained by **Rich et al. (2014)** who showed that TLR-2 acts as a sensor where it recognizes pathogens and is activated only when the epithelium is disturbed.

The results of this work showed that, TLR-2 was expressed mainly in cytoplasm of keratinocytes, however, membranous expression and sporadic nuclear expression were also detected in of low and high risk OED groups. This is consistent with **Ng et al.(2011)** who declared that TLR-2 was primarily localized to cytoplasm and nuclei of oral keratinocytes cells. Moreover, Rich et al. (2014) illustrated that sometimes membranous expression of TLR-2 was seen. The increase of TLR-2expression in high risk OED group in this study was agreeable with the results of **Huhta et al. (2016)** who noticed nuclear and cytoplasmic TLR-4 expression in oesophageal dysplasia and cancer which was associated with metastasis and poor prognosis.

The cytoplasmic expression could be explained by the fact that TLR-2 is an integral component of Golgi apparatus as well as phagocytic vesicle membrane present in the cytoplasm **(Donaldson and Lippincott-Schwatz, 2000).** This is in addition to the structure of TLR-2 which has a cytoplasmic domain **(Akira et al., 2001).**

The membranous expression of TLR-2 in this work could be attributed to that TLR-2 acts as a cell surface receptor for cytokines (IL-10 and IL-6)**Tang et al., (2016).**

The nuclear localization of TLR-2 observed in this study could be explained according to **Yang et al. (2014)** who illustrated that TLR-2 promoted the transcription of genes related to angiogenesis and invasion, such as VEGF-C and MMP-9. Acting as a transcription factor, this could explain its translocation to the nucleus and binding to the DNA from where it performs its function.

Some cases of low and high risk OED in this work showed an accentuated TLR-2 immunoexpression around the nuclear membrane. This is in accordance to **Osman et al. (2004)** who stated that TLR-2 is highly expressed in a perinuclear region close to the Golgi complex associated with microtubules. They explained that high focal concentration of TLR-2 immunoexpression at the perinuclear region is due to the microtubules suggesting that the microtubules serve as transport tracks for TLR-2 vesicles.

In this work inflammatory cells, endothelial cells and fibroblasts showed TLR- 2 immunoexpression. This is in accordance to **Chang (2010)** who detected TLRs in T and B cells as well as endothelial cells, epithelial cells and fibroblasts.

In this study, low risk OED showed TLR-2 immunoexpression in the lower1/3 of the epithelium, while high risk dysplastic epithelium showed TLR-2 immunoexpression extending to the upper two thirds or including almost all layers of the dysplastic epithelium showing a more obvious expression in the basal layers. This finding is following and confirming the histological grading criteria of epithelial dysplasia where in low risk OED the dysplastic changes are localized to the basal 1/3 of the epithelium, while in moderate and severe dysplasia (high risk), the dysplastic change extend to involve more than half of the epithelium and may involve the whole
thickness of the epithelium.

In the current study, statistical analysis revealed that the area percent of TLR-2 immunoexpression in low and high risk OED groups was significantly greater compared to the control group. This is in accordance with **Huhta et al. (2016)** who illustrated that TLR-1,2,4 and 6 immunoexpression were greater in oesophageal dysplasia compared to control normal mucosa. This could be due to the endogenous damage-associated molecular patterns (DAMPs) which are released from keratinocytes and entered a potentially neoplastic phase **(Sato et al., 2009).** These DAMPs activate the TLR-2 in dysplastic keratinocytes.

Moreover, in this work area percent of TLR-2 immunoexpression in high risk OED was significantly greater compared to the low risk OED. This finding is in agreement with (Pimentels-Nunes et al. 2011) who illustrated that TLR-2 in severe gastric dysplasia was significantly higher than mild gastric dysplasia. This could be due to the more extended epithelial involvement (more than half of the epithelial thickness) in high risk OED group.

The significant difference in area percent values of TLR-2 immunoexpression between oral dysplasia groups and control group in this experiment could allow the use of TLR-2 as a marker in evaluation of OED which could prevent mis-diagnosis of OED from questionable oral epithelium thus leading to proper treatment.

MCs have long been considered to play a specific role in pathophysiology of many diseases. Release of MC mediators has been thought to contribute to tissue injury and inflammation. Recent data have shown that inflammation is a critical component for tumor progression**(Mukherjee et al., 2009).**

In this respect, the current study attempted to use the TB stain in tissue sections to highlight MCs in normal and dysplastic tissue of rat buccal mucosa (Ingaleshwar et al., 2016). The mean number of MCs was calculated for high and low risk OED groups and compared with the control group. Results of the present work illustrated that normal rat buccal mucosa showed few MCs which were located deep in the connective tissue. This finding is consistent with **Telagi et al. (2015)** whose study showed a few number of MCs in normal oral mucosa compared to OED.

The role of MCs in oral dysplasia has been investigated by many researchers who showed a significant increase in the number of MCs as the tissue progressed from normal tissue to dysplastic or cancerous **tissue (Mohtasham et al., 2010, Telagi et al., 2015 ,Veda and Vinay, 2015 and Ramsridhar and Narasimhan,2016). The previous finding could be explained according to Lotze et al. (2007) and Sheu et al. (2009)** who illustrated that normal keratinocytes have not undergone genetic mutations leading to alterations of protein products and/or chromosome structure, thus they did not release DAMPs that activate MCs.

In this experiment, low and high risk OED groups showed MCs infiltrating the connective tissue directly beneath the dysplastic epithelium. This finding is in accordance to **Coussens et al. (1999)** who illustrated that MCs within dysplasia were juxtaposed tightly to the epithelium and basement membrane. These are the sites of active extracellular matrix remodeling and MCs contribute to extracellular matrix degradation and remodeling by releasing two MC specific serine proteases, a tryptase, a chymase, as well as progelatinase B.

Moreover, in this work, MCs in high risk OED were significantly higher than low risk OED and normal buccal mucosa. This is consistent with **Ramsridhar and Narasimhan (2016)** who showed an increase in MCC from normal oral mucosa to mild dysplastic leukoplakia to severe dysplastic leukoplakia and SCC. The author suggested that chemo attractants that are induced by tumour cells or normal connective tissue cells as a response to the tumor can be a reason for gradual increase in MCC from normal mucosa to OED and OSCC.

Therefore, this significant increase in the mean number of MCs as the normal tissue progresses into dysplasia allows the use of MCC as an indicator of disease progression which could have an important impact as regards management of dysplasia.

Finally, in this work, the increase in mean MCC is associated with an increase in TLR-2 immunoexpression which could be considered an early event of OED. This finding is consistent with Takeda and Akira (2005) who showed that the TLRs are expressed by MCs, macrophages and dendritic cells.

V. Conclusions

In respect to the previous results and discussion it can be concluded that:

- TLR-2 molecular biomarker plays a role in detection and diagnosis of low and high risk epithelial dysplasia.
- Theimmunoexpression of TLR-2 and the pattern of distribution of each positive immunoreaction may help in diagnosis and prognosis of (PMOC).
- Number ofMCs is correlated and directly proportional to the degree of OED
- Theimmunoexpression of TLR-2 was significantly correlated with MCC in the studied groups.

VI. Recommendations

It is recommended to:

- Perform further studies on the role of TLR-2 in oral premalignant and malignant epithelial lesions.
- Study the correlation between TLR-2 and other inflammatory cells.
- Draw further attention and perform studies directed to possible promising therapeutic role of TLR-2 and MCs in PMOC and OSCC.

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