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Immunohistochemical expression of p53 and Bcl2 in varying grades of Oral Epithelial Dysplasia

Abstract

Objective & Aim: The study was carried out to determine the frequency of p53 protein and Bcl2 gene expression in oral dysplastic lesions of varying grades. **Materials & Methods:** 120 paraffin blocks of oral epithelial dysplasia (OED) were retrieved from I.T.S dental college hospital and research centre Greater Noida. The immunohistochemical marker p53 and Bcl2 was applied using Streptovidin-Biotin technique. Staining and intensity of the marker was recorded and frequencies were compared in both the lesions. **Results:** Bcl2 gene was proven to be better marker than p53 protein in diagnosing the dysplastic lesions.

Key Words

Oral epithelial dysplastic (OED); p53 protein; Bcl2 gene

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INTRODUCTION

Oral epithelial dysplastic lesions, especially so called pre-cancers are among the most challenging and intricate topics of head & neck pathology.[1] Oral epithelial neoplastic lesions comprise a continuum from mild dysplasia to carcinoma with the specific criteria of the oral epithelial changes defining each stage along the continuum.[2] Lack of uniformity among the pathologists in risk assessment of a lesion has lead to much modification to improve the positive predictive value and to increase the reproducibility. Many molecular markers functioning as elements of cell cycle control have been recognized such as p53 and Bcl2. Aberration of p53 gene is the most common genetic alteration in human cancer. It has a central role in genome stability and cell cycle regulation and also its function is abrogated in most human cancers and in case of oral mucosa also in the pre-invasive stage. p53 prevents the accumulation of DNA damage in cells by activating transcription of genes involved in G1 arrest, particularly the p21/waf1/cip-1 gene; thus blocking the entry of damaged cell into the s phase of cell cycle and allowing DNA repair before replication, in addition,p53 is a pro-apoptotic gene, i.e., it triggers apoptosis of cells with reverse DNA damage by up regulating Bax, both the mechanisms provide a barrier to the propagation of mutated cells and consequently p53 has been designated as "the guardian of the genome".[3] The Bcl2 proto-oncogene is an important member having a relevant role in tumour development by inhibiting apoptosis.[8] Past studies have used p53 and Bcl2 as specific adjuncts to the grading of epithelial dysplasia. Thus, in the present study an attempt has been made to evaluate the use of p53 and Bcl2 as a reliable diagnostic marker in grading of oral epithelial dysplasia

MATERIALS & METHODS

A total of 120 subjects, 20 each of mild, moderate and severe dysplasia both for p53 protein and bcl2 gene were selected by random sampling. All the samples were retrieved from ITS dental college hospital and research centre Greater Noida between January 2013 and January 2014. Histopathological features of the selected samples were reviewed from freshly prepared H & E sections. Diagnosis of the dysplasia was made according to the criteria given by WHO. Biogenex labelled streptovidin (LSAB) with system peroxidase kit was used.Two sections each of 3 µm thick sections were made and taken on

poly-L-lysine coated slides.Sections were air-dried at room temperature. The sections were deparaffinised by heating on the slide warming table at 60 degrees celsius for 15 to 20 minutes and then passed through 2 changes of xylene for 5 minutes each. Sections were rehydrated by taking them through 3 changes of 100% alcohol for 5 minutes each, followed by 90% & 70% alcohol for 5 minutes each. Sections were then brought down to water for 5 minutes. The deparaffinised tissues were placed in tanks containing Tris buffer (retrieval solution) at ph 9 and brought to a boil in an EZ antigen retrieval machine in 2 cycles. First cycle at 85 degree celcius for 10 minutes and second cycle 100 degree celcius for 5 minutes. The slides were cooled to bring to room temperature for 30 min. Slides are then washed in pbs for 5 minutes. Sections were blocked with endoperoxidase block for 10 minutes, washed twice in PBS for 5 minutes. Sections were treated with power block for 5 minutes. Sections were then treated with primary antibody for 1 hour to 1 hour 15 minutes, again washed with PBS for 5 minutes. The sections were then treated with super enhancer for 20 minutes. The sections were washed with PBS and then treated with SS labelled poly HRP substrate (secondary antibody) for 20 minutes. Sections were covered with freshly prepared substrate chromogen solution for 10 minutes. The slides were then washed with phosphate buffer saline (PBS) and counterstained. Slides were immersed in Harris hematoxylin for 30 seconds to 1 minute, washed gently under running water and then washed with distilled water and allowed to dry in air at room temperature. The sections were dehydrated, dipped in xylene and were later mounted using DPX, a non-aqueous permanent mounting medium. Coverslip was placed and the slides were interpreted using research microscope. A histopathologically confirmed case of oral squamous cell carcinoma was taken as a positive control for expression of p53 and for Bcl2 normal human lymph node served as a positive control whereas negative control was an immunohistochemically stained section in which the primary antibody (anti p53- mouse monoclonal and Bcl2) has been omitted but included all other steps of immunohistochemistry. All the slides were viewed under the light microscope. The positively stained *Bcl*-2 cells and p53 nucleus will take up brown color (hotspots). 100 positive cells will be counted in these 10 pictomicrographs and the percentage of the mean will be taken and will be assigned one of the 5 categories

- <5% cells stained: Category 0
- 5-25% cells stained: Category 1
- 25 to 50 cells stained: Category 2
- 50 to 75 cells stained : Category 3
- 75 to 100 cells stained : Category 4

Staining of each slide was scored according the percentage of cells stained in each case and they were graded according to the grading system given by kowichi Nakagawa[5]

- Strongly (intensely) positive :more than 50 % cells stained (category 3 & 4)
- Moderately positive: 25>50% cells stained : (category 2)
- Weekly positive :5>25% cells stained (category 1)
- Negative staining: less than 5% cells stained: (category 0)

The mean percentage of p53 and Bcl2 count in mild, moderate and severe dysplasia were calculated value is calculated by one way analysis of variance (ANOVA). Statistically, comparison between p53 and Bcl2 was done according to Post hoc pair wise comparison by Tukey's test. The mean difference is significant at the 0.005 level.p53 and Bcl2 stained slides were categorized into 5 different categories depending on the mean percentage of cells and the p value was calculated using chi square test and were tabulated. Intensity of p53 & Bcl2 staining according to Nakagawa was calculated using chi square test and tabulated. Correlation between degree of staining in p53 and Bcl2 were calculated statistically using spearman correlation coefficient and p value was calculated. The summary of the immunostaining data and staining intensity are given of p53 protein and Bcl2 gene in the charts.

RESULTS

The summary of the immunohistochemical data and staining activity of p53 ad Bcl2 in oral dysplasia is given in Tables 1. All the values for mean p53 & Bcl2 count for mild, moderate & severe dysplasia are expressed in terms of mean percentage of cells.Standard deviation for mean value is also calculated for each dysplasia. One way Analysis of variance test was applied and a significant difference was observed between mild, moderate and severe dysplasia. Photomicrographs of the immunohistochemical Bcl2 staining in mild,moderate and severe dysplasia in varying grades of oral Epithelial dysplasia (Fig. 1, Fig. 2 & Fig. 3). Photomicrographs of the

Table 1

Group (Grade of dysplasia)	Expression of p53		Expression of Bcl2	
	Mean % age of stained cells	Standard deviation	Mean % age of stained cells	Standard deviation
Group I	23.94	3.32	42.61	10.11
Group II	48.87	3.89	50.74	16.85
GroupIII	68.14	10.02	85.13	7.08
P value (Predictive value using One way analysis)	**<0.001, S**		**<0.001, S**	
Post hoc (pairwise comparison by Tukey's test)	Mild<Moderate<Severe		Mild<Moderate<Severe	

Table 2

Group (Grade of dysplasia)		**Category wise distribution for p53 staining**				**Main Category**
		1 (5-25% cells Stain)	**2 (25-50% cells Stain)**	**3 (50-75% cells Stain)**	**4 (75-100% cells Stain)**	
Mild	No. cases Showing staining	16	4	0	0	Category 1
	% age of cases Showing staining	80.0%	20.0%	0.0%	0.0%	
Moderate	No. cases Showing staining	0	15	5	0	Category 2
	% age of cases Showing staining	0.0%	75.0%	25.0%	0.0%	
Severe	No. cases Showing staining	0	0	15	5	Category 3
	% age of cases Showing staining	0.0%	0.0%	75.0%	25.0%	
P value		<0.001, S				

Table 3

Group		**CATEGORY For Bcl2**				**MAIN CATEGORY**
		1 (5-25% cells)	**2 (25 -50% cells)**	**3 (50-75% cells)**	**4 (75-100% cells)**	
Group I	Number of cases stained	3	17	0	0	Category 1
	% age of case stained	15.0%	85.0%	0.0%	0.0%	
Group II	Number of cases stained	0	5	15	0	Category 2
	% age of case stained	0.0%	25.0%	75.0%	0.0%	
Group III	Number of cases stained	0	0	0	20	Category 3
	% age of case stained	0.0%	0.0%	0.0%	100.0%	
Pvalue (Chi square test)		<0.001, S				

immunohistochemical p53 staining in mild, moderate and severe dysplasia in varying grades of oral Epithelial dysplasia (Fig. 4, Fig. 5, Fig. 6). Category wise distribution of p53 stained cases according to the mean percentage of cells stained in Mild, Moderate & Severe dysplasia cases done using Chi square test is given in Table 2. Categorization of Bcl2 stained cases according to the mean percentage of cells stained in Mild, Moderate & Severe dysplasia cases done using Chi square test is given in Table 3. Distribution of p53 stained cases of Mild, Moderate and Severe cases according to the intensity of staining applying Chi square test is given in Table 4. Distribution of Bcl2 stained cases of Mild, Moderate and Severe cases according to the intensity of staining using Chi square test is given in Table 5. Correlation between p53 & Bcl2 staining and degree of dysplasia is given in Table 6.

DISCUSSION

Oral squamous cell carcinoma is usually preceded by dysplastic or precancerous lesion .p53 tumour

Table 4

Groups		Intensity of staining for p53			Predominant staining intensity
		Weak Staining	Moderate Staining	Intense Staining	
Group I (Mild)	Number of cases Showing staining	16	4	0	Weak Staining
	% age of cases showing staining	80.0%	20.0%	0.0%	
Group II (Moderate)	Number of cases Showing staining	0	15	5	Moderate Staining
	% age of cases showing staining	0.0%	75.0%	25.0%	
Group III (Severe)	Number of cases Showing staining	0	0	20	Intense Staining
	% age of cases showing staining	0.0%	0.0%	100.0%	
P value (Chi square test)				<0.001, S	

Table 5

GROUP		Intensity of staining for Bcl2			Predominat staining Type
		Weak staining	Moderate staining	Intense staining	
Group I (Mild)	Number of cases showing staining	3	17	0	Moderate staining
	% age of cases showing staining	15.0%	85.0%	0.0%	
Group II (Moderate)	Number of cases showing staining	0	5	15	Intense staining
	% age of cases showing staining	0.0%	25.0%	75.0%	
Group III (Severe)	Number of cases showing staining	0	0	20	Intense Staining
	Number of cases showing staining	0.0%	0.0%	100.0%	
P value (Chi square test)				<0.001, S	

Table 6

Degree of dysplasia	Spearman correlation coefficient, p value
Mild	rho = 0.651
	P<0.001, S
Moderate	rho = 0.500
	P=0.001, S
Severe	rho = 0.775
	P<0.01, S

suppressor gene and its proteins are altered very early in the process of carcinogenesis. The role of Bcl2 family members in the development of cancer comes from several studies. Retrospective studies have suggested its role in up regulation of Bcl2 in early carcinogenesis. Thus in the present study, p53 and Bcl2 were used to evaluate their experience in varying grades of oral Epithelial Dysplasia. Sixty histopathologically proven cases of dysplasia were taken and subsequently stained with Bcl2 and p53.

Positive stained nucleus (brown in color) for p53 and positive stained cytoplasm (brown in color) for Bcl2 were counted, recorded and tabulated. The results of our study showed a mean p53 count of 23.94± 3.32 for mild dysplasia, 48.87±3.89 for moderate dysplasia and 68.14±10.02 for severe dysplasia. 5-25% of cells stain in 80% of cases of mild dysplasia, 25-50% of cells stain in 75% cases of moderate dysplasia, 50-75% cells stain in 75% cases of severe dysplasia and more than 75% cells stain in 25% cases of severe dysplasia. The above mentioned finding was further supported by one way Analysis of variance (ANOVA) test which showed a statistically significant difference in p53 count among the three groups. Thus a progressive increase in p53 count from mild-moderate to severe dysplasia was observed as in support by Girod SC and Polkowski. However the mean p53 count for the three groups in the present study was more than in literature.[6] The reason for such great variability could be because of many factors, including case selection, sample size, number of cells counted, evidence of reproducibility and methods of statistical analysis used.Other possible variables include anatomical site, fixation and antigen retrieval technique.[7,8] For p53 for the staining 80%

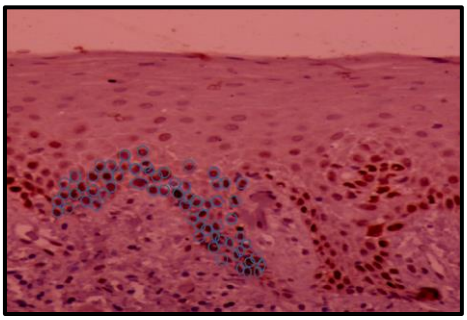

Fig: 1: Photomicrograph of Bcl2 stained tissue showing number of cells stained /200 cells in Mild Epithelial Dysplasia(X 40)

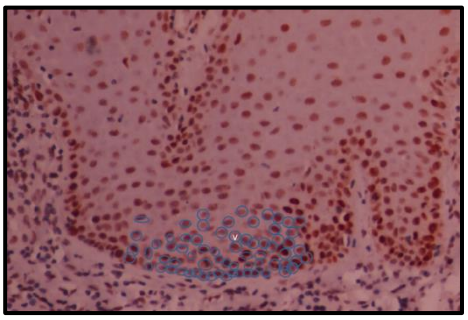

Fig 2: Photomicrograph of Bcl2 stained tissue showing number of cells stained /200 cells in Moderate Epithelial Dysplasia(X 40)

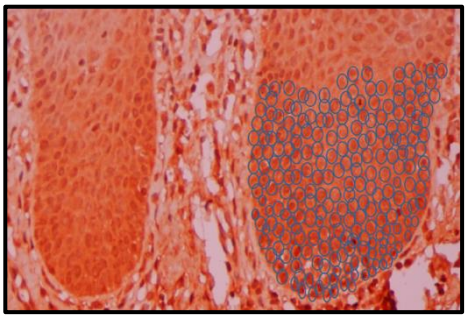

Fig. 3: Photomicrograph of Bcl2 stained tissue showing number of cells stained /200 cells in Severe Epithelial Dysplasia(X 40)

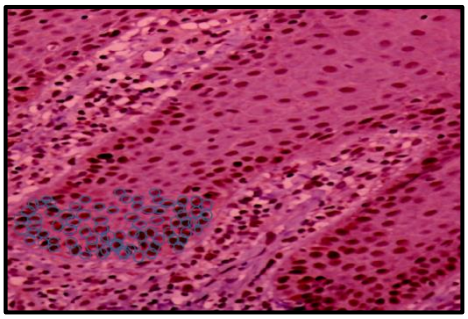

Fig. 4: Photomicrograph of p53 stained tissue showing number of cells stained/200 cells in mild Epithelial Dysplasia(X 40)

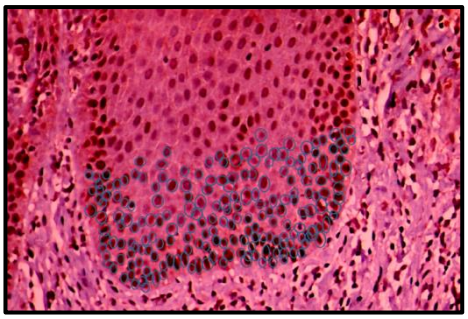

Fig 5: Photomicrograph of p53 stained tissue showing number of cells stained/200 cells in moderate Epithelial Dysplasia(X 40)

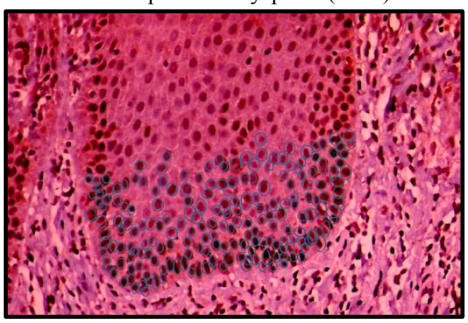

Fig. 6: Photomicrograph of p53 stained tissue showing number of cells stained/200 cells in severe Epithelial Dysplasia(X 40)

of mild dysplasia cases exhibit weekly positive staining,75% of moderate dysplasia cases exhibits moderately positive staining and 100% cases of severe dysplasia exhibits intense staining. Wood *et al*.,[9] Girod *et al*.,[10] found a significant correlation between p53 expression and grade of dysplasia,p53 count increase with increasing the grade of dysplasia. Significantly higher number of p53 positive cells was found in lesions having moderate and severe dysplasia. The findings of our study are in concurrence with this study. The expression of p53 system in epithelia with dysplasia could simply indicate p53 system in functioning correctly and is active and might be repaired or brought to apoptosis or might enter cell cycle of malignant transformation. Our study showed a clear correlation between grades of Epithelial Dysplasia however, studies by Regezi and Cruz,[3,11] could not find a clear correlation between grades of Epithelial Dysplasia and percentage of p53 positive cells in oral premalignant lesions which is opposite to our study. This discrepancy found among studies may be due to subjectivity in the assessment of dysplasia, differences in the population studied or sampling differences. Under normal condition, the basal layer is the site in the epithelium where cell proliferation is generally observed; the suprabasal layer show evidence of maturation. This implies that the basal and suprabasal expression of p53 responds to accumulation of normally functioning wild type of protein. It is a normal physiologic response to slow down the cell cycle at G1 phase to allow the repair of damaged DNA. Whereas suprabasal expression could indicate the presence of

mutated protein in DNA damaged proliferative cells. Thus supra basal immune staining of p53 protein could behave as an objective marker for the presence and severity of epithelial dysplasia, since it would indicate the presence of proliferative cells in epithelial layers at a site where only differentiated cells should have existed. Thus basal and suprabasal expression of p53 probably have distinct implications.[12] The present study showed a predominant intense staining in the basal layer & suprabasal layer in mild, moderate & severe dysplasia. Shin DM *et al*., reported a quantitative image analysis which demonstrates not only a gradual increase in the amount of p53 expression as tissue abnormalities progressed but also a topological change in expression. p53 expression was present in basal layer in normal tissue but was expanded to parabasal & superficial layer in hyperplasia and dysplasia. They concluded that p53 expression can be altered in very early phases of head & neck tumorogenesis and it can be used as an intermediate biomarker in chemoprevention trials.[13] Various studies have shown various amount of positivity for p53 in oral epithelial dysplasia ranging from 45%,[13] 68%,[14] 75%,[15] to 100%.[16] The results of our study go in accordance with study by Ravi *et al*.[16] Cruz IB,[3] Kaur J. Srivastava.[17] suggested that the clear expression of p53 above the basal layer is an early event in oral carcinogenesis and an indicator of a developing carcinoma, even preceding morphological tissue alterations. p53 is strongly recommended in conjugation with histological parameters, to increase the sensitivity of detection of cases that will progress to carcinoma. Gonzalez and Naglaa Fathy[18] in year 2002 commented that p53 expression does not behave as an objective marker for the presence or severity of Epithelial Dysplasia. Warnakulasuriya KA,[12] Johnson NW suggested that p53 gene mutations are commonly involved in oral cancer but are neither sufficient nor necessary for the development of malignancy. It is possible that its presence can be used as a marker of risk in a high proportion of malignant & potentially malignant oral lesions. Shahnavaz SA concluded in his study that there is inconsistent relationship between gene mutations and the level of p53 protein staining by immunohistochemistry. p53 gene mutations seem to occur relatively late and they are associated with transformation to the invasive phenotype. Trere[19] for the first time in human breast showed that the status is related to the ribosome's biogenesis rate. This biogenesis rate is a consequence of the loss of the inhibitory affects of p53 and pRB (retinoblastoma gene) on RNA polymerase I activity. This up regulation of rRNa synthesis may be considered a mechanism by which dysplastic /cancer cells with deregulated cell cycle progression can always reach, at the end of each cycle, a size large enough to divide without ever becoming smaller until they can no longer proliferate. Thus in the light of this fact, a high ribosome biogenesis rate would be necessary for progression of tumours with alteration p53 and pRb functions. Pestov[20] and Rubbi[21] showed that exposure of cells to various forms of exogenous stress, eg. UV radiation, nucleotide depletion, heat shock, hypoxia etc., but also aberrant ribosome biogenesis may cause nucleolar stress sensor responsible for maintenance of low levels of p53, which are elevated as soon as nucleolar function is impaired. Under stress conditions that inhibit RNA polymerase I (pol I) transcripts, the structure of nucleolus is perturbed and scavenging proteins, including ribosomal proteins L5, L11 and L23, $p19^{ARF}$ or nucleophosmin, are released from nucleolus to the nucleoplasm,where they associate with MDM2 to inhibit its activity and thus stabilize p53. Similarly, in the present study, a steady increase was found in Bcl2 positive cells from mild-moderate to severe dysplasia, substantiated by ANOVA test which showed a statistically significant difference in Bcl2 count among the three groups. The results of our study showed a mean Bcl2 count of 42.61±10.11 for mild dysplasia, 50.74±16.85 for moderate dysplasia and 85.13±7.08 for severe dysplasia. 5-25% of cells stain in 15% of cases of mild dysplasia, 25-50% of cells stain in 85% cases of mild dysplasia & in 25% cases of moderate dysplasia.50-75% cells stain in 75% cases of moderate dysplasia and more than 75% cells stain in all the cases of severe dysplasia. Thus a progressive increase in Bcl2 count from mild-moderate to severe dysplasia was noted. The results of staining intensity for Bcl2 exhibited that in 85% of mild dysplasia case exhibited moderate staining, 75% of moderate dysplasia and all cases of severe dysplasia show intense staining. Our study goes in accordance with study done by Ravi *et al*., on expression of p53 & Bcl2 proteins in hyperplastic mucosa, dysplastic mucosa and invasive oral carcinoma, 80% of which states that severe Epithelial Dysplasia show intense immunoreactivity only 2 cases exhibited moderate reactivity. Oral

Epithelial Dysplasia show transformation rate of 13.8% to invasive carcinoma. Genetic events associated with the transformation into carcinoma are not well characterized, but alteration of tumor suppressor gene have been reported.[21] The importance of Bcl-2 expression in carcinogenesis is still controversial.[22] Ravi *et al*.,[16] Okazaki Y *et al*.,[23] demonstrates increased expression in dysplastic lesions. Few authors like Piatelli A,[24] Schoelch ML,[25] Staibano S *et al*.,[26] de Vicente JC *et al*.,[27] described little or no Bcl-2 expression in dysplastic lesions. In our study Bcl-2 expression increased with the increase in the grades of dysplasia, it goes in agreement with Ravi *et al*.,[16] Okazaki Y *et al*.[28] In the initial phases of carcinogenesis Bcl-2 act as an anti-apoptotic protein, favouring malignant transformation. As a cancer develops more Bcl2 molecules are present and Bcl-2 is then cleaved acting as a pro-apoptotic protein, which has already been described by Cheng *et al*.[29] This explains the conflict between the results generated by different authors for the expression of Bcl-2 in dysplasia.[16,24,28,29] Singh *et al*., first attempted to correlate Bcl2 expression levels to the grade dysplasia. He demonstrated a direct correlation between the expression of Bcl2 to the involved thickness of epithelium in Mild, Moderate and Severe dysplasia. Marked immune reactivity involving the entire thickness of the epithelium with diminishing reaction to superficial layers was observed in our case which goes in accordance with finding of Singh *et al*., Bronner *et al*., Lai Fashu *et al*., Brichell *et al*. This may be due to the inhibition of apoptosis and offer an advantage to the rapidly growing tumor by slowing down the cell death rate.[4] Our study support the hypothesis that enhanced Bcl2 expression prevents apoptosis and is a common early event in tumorigenesis and play an important role in the development of some tumors of epithelial origin. Bcl2 expression appears to be altered to varying degrees in oral mucosal dysplasia.[31] Few studies employing it on dysplasia show Bcl2 expression in 16%, 37% and 81% of dysplastic tissue[32] but our study shows higher percentage of expression than suggested in the literature. We noticed interesting correlation between number of cells stained and the intensity of stain as we increased the grades of dysplasia. This goes in accordance with the study done by Sulkowska *et al*.,[29] who observed a direct correlation between the expression of p53 and Bcl-2 and the degree of Epithelial Dysplasia. Statistically Bcl2 showed more intensity of staining and increased number of stained cells as we increased the grading in Epithelial Dysplasia when compared with p53 staining. For mild dysplasia Spearmans correlation coefficient, rho in Mild dysplasia is 0.651, for Moderate dysplasia Spearmans correlation coefficient, rho is 0.500 and for severe dysplasia rho is 0.775 and p value is less than 0.01 for all the grades of dysplasia.

CONCLUSION

The present study is focussed on the expression of the p53 & Bcl2 onco-protein in 120 histological sections of varying grades (mild, moderate and severe) of Oral Epithelial Dysplasia, and a comparison was also done between both types of immunohistochemistry markers. The validity of study with respect to Bcl2 and p53 expression is quintessentially done from their point of view for thematic approach to deal with the prediction of probability of malignancy. Thus, it would be important for studies like this to analyze these cases from a prospective manner, which would specifically highlight graded values achieved for p53 and Bcl2 with respect to mild, moderate and severe dysplasia.

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