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Original Research Article.

Immunohistochemical Expression of JAK-P, STAT3 & BCL-2 in Oral Squamous Cell Carcinoma with and Without Lymph Node Metastasis And in Verrucous Carcinoma

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ABSTRACT

Objective: Inappropriate activation of JAK/STAT pathway and BCL-2 occurs with high frequency in human cancers and is associated with cancer cell survival and proliferation. However, its role in oral squamous cell carcinoma (OSCC) with and without lymph node metastasis and in verrucous carcinoma (VC) is still debatable.

Methods: By immunohistochemistry, we analysed the expression of phosphorylated JAK-2 (pJAK-2), STAT-3 and BCL-2 in resection specimens of OSCC with and without lymph node metastasis (30 cases each), in Verrucous carcinoma (30 cases) and in normal mucosal margins (20 cases). We also compared their respective immunohistochemical expression between OSCC, VC and in normal oral mucosa.

Results: We found that pJAK-2 and STAT-3 expression was not detectable in normal mucosa however JAK/STAT pathway was involved in 66% of OSCC and 23% of Verrucous carcinoma.Bcl-2 expression was noted in 23% of OSCC and 6.6% of Verrucous carcinoma.

Conclusion: Oral cancer is the third most common in India with most common age of presentation being 4th-5th decade. Immunoexpression of bcl-2 revealed a bad prognostic marker as it showed inverse correlation with tumor differentiation in OSCC. Majority of these tumors show involvement of JAK-STAT pathway in the tumor development and progression which accounts for 66.6% in OSCC and 23% in VC. Further clinical trials and molecularly targeted research on JAK-P, STAT3 and BCL-2 is required to improve the treatment, patient care and prognosis as it is the major pathway involved in development of OSCC.

Keywords: Immunohistochemical Expression, JAK-P, STAT3, BCL-2, Squamous Cell Carcinoma, Lymph Node Metastasis, Verrucous Carcinoma.

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INTRODUCTION

Various pathways have been involved in head and neck squamous cell carcinoma (HNSCC) such as EGFR, RAS/MAPK/ERK pathway, PI3K/AkT pathway, mTor pathway. However, studies on activation of JAK-STAT pathway have been limited in HNSCC. Poorly differentiated subsets of HNSCC, resistant to EGFR targetted therapies such as erlotinib and geftanib and PI3K/AKT targeted therapies have not found to be effective.[1-5] Hence, development of newer and effective regimens in HNSCC is the need of hour.

Hence, in this study we aim to study JAK/STAT pathway in HNSCC and Verrucous carcinoma. Along with it, major antiapoptotic downstream regulator BCL-2 is also studied.

METHODS AND MATERIALS

Patients (*n* = 90; 60 cases of OSCC+30 cases of VC), who were treated at All India Institute of Medical Science, New Delhi, between September 2013 – October 2015 (duration – 2 years) which included 78 men and 12 women, ranging in age from 26 to 79 years (mean- 48 years) were included in this study. The medical records of these patients were reviewed to obtain information regarding the clinicopathological characteristics of the lesions, including their localization, size, stage and degree of differentiation. The expression of Bcl-2, JAK-P and STAT-3 in 20 sections of normal mucosa was also investigated as normal control.

For our convenience we divided the cases (n=90) into 3 groups:

Group I: OSCC without lymph node metastasis (n=30);

Group II: OSCC with lymph node metastasis (n=30);

Group III: Verrucous carcinoma (n=30)

Immunohistochemistry

Serial 5 micron thick sections were cut from the selected representative paraffin embedded tissue blocks and overlaid on poly-L-lysine coated slides, deparaffinized in two changes of xylene for 5 minutes and one change of acetone for 1 minute,

followed by rehydration in decreasing concentrations of alcohol (95% ethanol for 3 minutes, 70% ethanol for 3 minutes, and distilled water for 1 minute). For all the immunostains (bcl-2, jak-p and stat 3), antigen retrieval was done by heating the sections immersed in citrate buffer at pH 6 inside a 800 watt microwave oven in full power for 30 minutes.

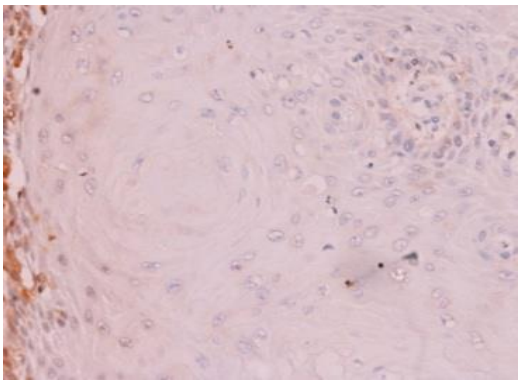
To diminish the nonspecific staining (endogenous peroxidase activity) each slide was treated with methanol containing 4% hydrogen peroxide. Sections were then overlaid with adequate amount of appropriately diluted primary antibody followed by overnight incubation at 4° C in a humid chamber. For pJak2, *Cell Signaling source* at a dilution of 1:400 with endometrial carcinoma as a positive control, stat3, *Spring Bioscience source* at a dilution of 1:200 with breast carcinoma as a positive control and for bcl-2, *Spring bioscience sourceSP66 clone* at a dilution of 1:400 with tonsil as a positive control was used. It was followed by 3 washings (5 minutes each) in TRIS-HCL buffer, peroxidase conjugated streptavidin was applied to cover the specimens and incubated at room temperature for 30 minutes. Sections were then covered with substrate chromogen solution freshly prepared by dissolving 1 mg of 3, 3' - diaminobenzidine tetra hydrochloride (DAB) in 1ml of DAB substrate (ScyTek laboratories, USA). The slides were incubated at room temperature for 5-10 minutes under microscopic control till the optimum development of brown coloured peroxidase reactant product. After rinsing in distilled water, the sections were counterstained with Harris haematoxylin followed by mounting with DPX as mounting media. Following this, immunostaining was scoredon the basis of percentage immunopositivity and staining intensity. The samples were initially graded based on the percentage of positively stained cells: 0, positively stained cells <10%; 1, 10%–30%; 2, 30%-50%; 3, 50%--70% and 4, 70%--100%. The samples were further graded based on intensity: 0, negative; 1, weak yellow; 2, yellow; 3, dark yellow or brown. Overall, the samples were scored based on the grades of both percentage and intensity:

SCORE ≥3; Taken as POSITIVE

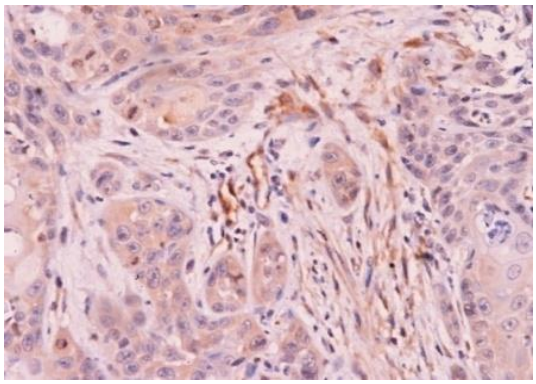
SCORE <3; Taken as NEGATIVE

Statistical Analysis

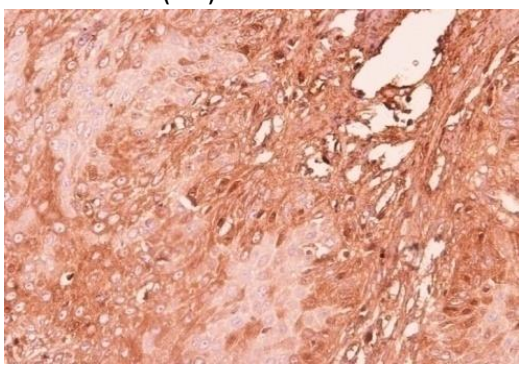
Data was analyzed using STATA 11.2 (Stata Corps, Texas, USA) and appropriate parametric and non-parametric tests were employed according to the studied variable. A p-value <0.05 denoted a statistically significant difference.



bcl-2 (40X): Well differentiated



bcl-2 (40X): Moderately differentiated



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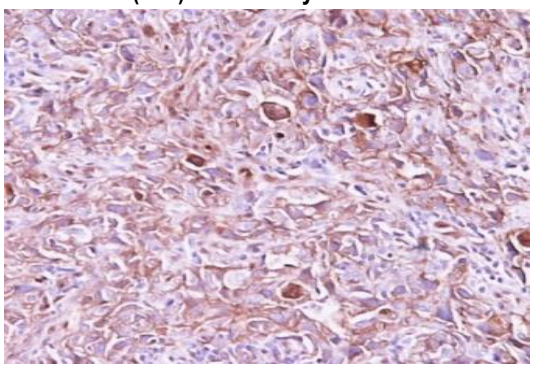
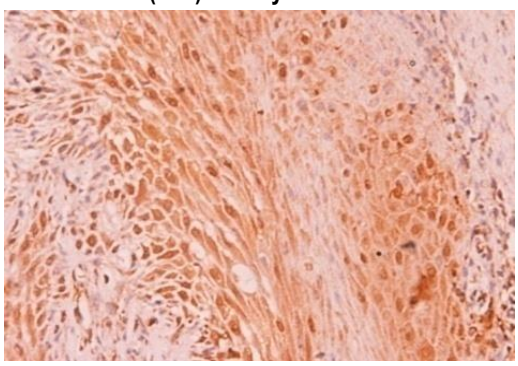
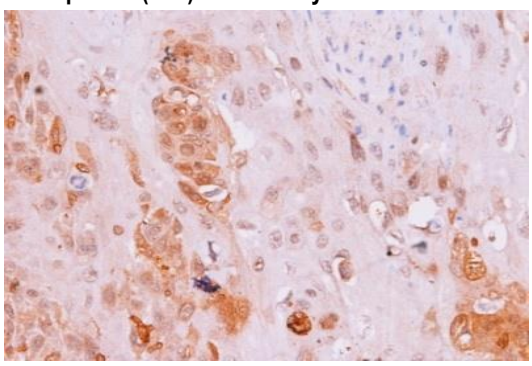


pJak-2 (40X): Moderately differentiated

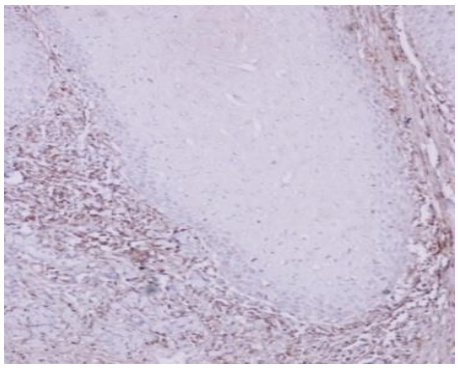


Stat 3 (40X): Moderately differentiated (Nuclear)

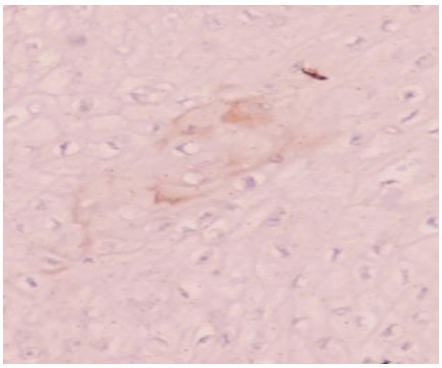


Stat 3 (40X): Moderately differentiated (Cytoplasmic)

Figure 1: bcl-2, pJak2 and Stat3 Immunoexpression in OSCC



bcl-2 (10x)



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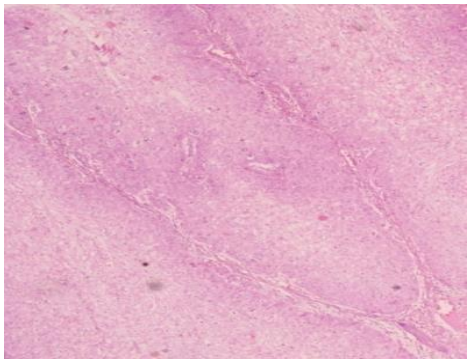
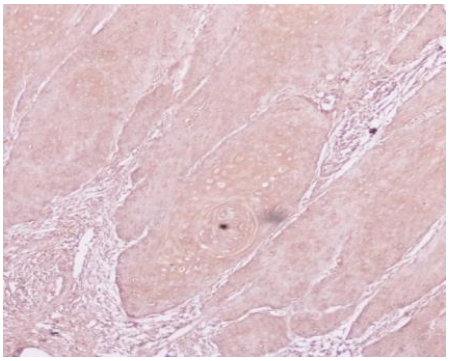
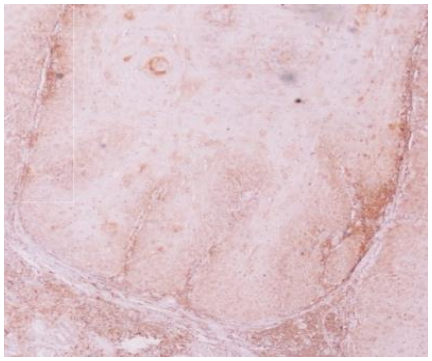


H&E (10x)



pJak2 (20x)



Stat 3 (20x)

Figure 2: Verrucous Carcinoma

RESULTS

The expression and cellular distribution of pJAK-2, BCL-2 and STAT-3 in HNSCC tumours were examined using immunohistochemical staining. pJAK-2 and STAT-3 expression was not detectable in normal oral mucosa. In tumour cells, cytoplasmic and nuclear expression was seen for STAT3 protein, cytoplasmic and membranous expression was seen for pJak2 while only cytoplasmic expression was observed for bcl-2 protein. However, bcl-2 expression was observed in basal cells of normal mucosa and in full thickness of Verrucous hyperplasia. There was a male preponderance in both the groups with M:F ratio of 4.4:1 in OSCC (Group I+ Group II) and 24:1 in Verrucous Carcinoma (Group III) respectively. The most common site for OSCC (Group I+II) was gingivobuccal sulcus (47%) followed by tongue (35%), buccal mucosa (18%) and lip (2%). On the other hand most common site for Verrucous Carcinoma (Group III) was buccal mucosa (48%) followed by an equal incidence in gingivobuccal sulcus and tongue (each 20%). In OSCC (with and without lymphnode), bcl-2 expression is inversely proportional to the tumor differentiation, Group I, p-0.001; Group II, p- 0.001.

However, pJak2 and stat 3 protein did not reveal statistically significant correlation with tumor differentiation, Group I, p-0.31 & 0.137, Group II, p- 0.318 & 0.31. Immunohistochemical correlation between pJAK2 and STAT3 was found to be statistically significant in all three groups, Group I, p-0.026; Group II, p-0.002; Group III, p-0.03. However immunohistochemical correlation between stat3 and bcl-2 was found to be statistically significant only in Group I, p-0.006. No immunohistochemical correlation was found between pJak2 and stat3 expression in all the three groups. No statistically significant association was observed between pJak2, stat3 and bcl-2 immunoexpression with the clinicopathological parameters such as age, sex, size, site and grade of the tumor in both OSCC and VC. On Comparing OSCC (Group I+Group II) and Verrucous carcinoma (Group III), statistically significant difference was found between sex and size of the tumor as Verrucous carcinoma showed more male preponderance as compared to OSCC (Group I+II). Most Verrucous carcinoma cases were less than 4 cm while most OSCC (Group I+II) were more than 4 cm in size. Statistically significant difference of stat3 immunoexpression between OSCC and VC was seen as 88% of OSCC cases were positive as compared to VC in which only 30% cases were positive.

Table 1: Relationship of clinical variables and IHC markers (pjak2, bcl-2 and stat3) in Oral squamous cell carcinoma (Group I+II)

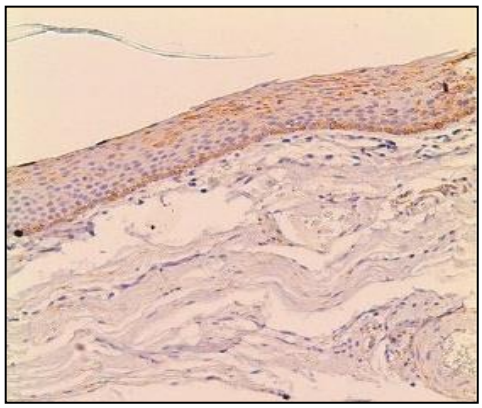
Variable	Total cases (n=60)	pJak2		p value	Stat3		p value	bcl- 2		p value
		L	H		L	H		L	H	
Male	49	16	33	**0.117**	14	35	**0.175**	43	6	**0.601**
Female	11	1	10		1	10		9	2	
≤ 40 yrs.	20	6	14		3	37	**0.741**	34	6	**0.591**
> 40 yrs.	40	11	29	**0.839**	2	18		18	2	
Gingivobuccal sulcus	27	7	20	**0.652**	2	26	**0.511**	26	2	**0.557**
Buccal mucosa	12	5	7		0	11		9	2	
Tongue	20	5	15		3	17		16	4	
Lip	1	0	1		0	1		1	0	
WDSCC	36	11	25		12	24	**0.321**	34	2	**<0.001**
MDSCC	15	2	13	**0.09**	2	13		10	5	
PDSCC	9	5	4		2	7		1	8	
≤ 4 cm	44	3	41	**0.481**	4	40	**0.725**	39	5	**0.457**
> 4 cm	16	2	14		1	15		13	3	

Table 2: Relationship of clinical variables and IHC markers (pjak2, bcl-2 and stat3) in Verrucous carcinoma (Group III)

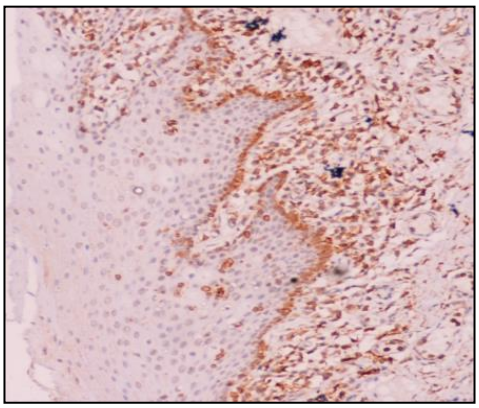
Variable	Total cases (n=30)	pJak2		p value	Stat 3		p value	bcl-2		p value
		L	H		L	H		L	H	
Male	29	16	33	**0.117**	14	35	**0.175**	43	6	**0.601**
Female	1	1	10		1	10		9	2	
≤ 40 yrs.	6	6	14		3	37	**0.741**	34	6	**0.591**
> 40 yrs.	24	11	29	**0.839**	2	18		18	2	
Gingivobuccal sulcus	10	5	5	**0.692**	7	3	**0.916**	10	0	**0.239**
Buccal mucosa	16	7	9		12	4		15	1	
Tongue	3	1	2		2	1		2	1	
Lip	1	1	0		1	0		1	0	
≤ 4 cm	24	3	41	**0.481**	4	40	**0.725**	39	5	**0.457**
> 4 cm	6	2	14		1	15		13	3	

Table 3: Comparison of OSCC (Group I+ Group II) versus Verrucous Carcinoma (Group III)

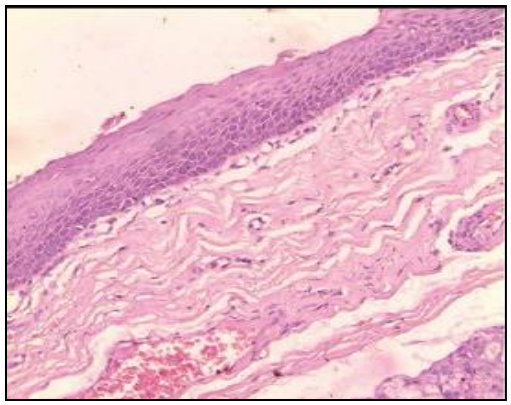
	OSCC (n=60)	VC (n=30)	p value
Age: <40	20 (33%)	6 (20%)	0.188
>40	40 (67%)	24 (80%)	
Sex: Male	49 (81%)	29 (96%)	0.04
Female	11 (19%)	1 (4%)	
SIZE: <4 cm	22 (37%)	25 (83%)	0.001
>4 cm	38 (63%)	5 (17%)	
pJak2	42 (70%)	17 (56%)	0.210
stat3	53 (88%)	8 (30%)	0.001
bcl-2	14 (23%)	2 (6%)	0.05



bcl-2 (20x)



bcl-2 (40x)



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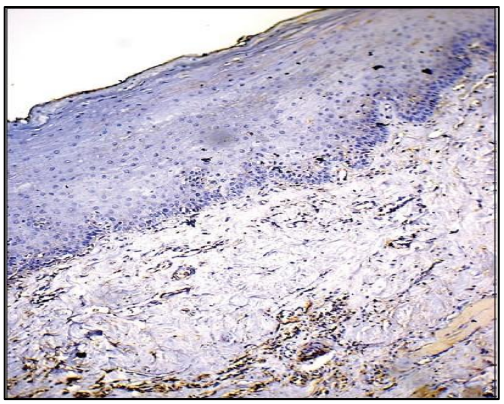
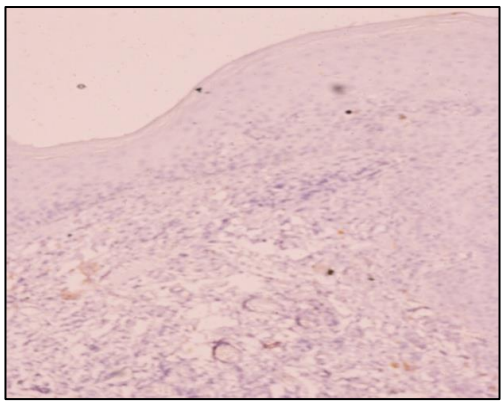


pJak2 (20x)



Stat 3 (20x)

Figure 3: pJak2, Stat 3 and bcl-2 in immunoexpression in normal mucosa

DISCUSSION

JAK/STAT pathway is detected in many human cancers. JAKs are a family of cytoplasmic tyrosine kinase – comprised of four members- jak1, jak2, jak3 and tyk-2.[6] STATs are a family of downstream transcription factors of JAKs and other kinases and include STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT 6.[7-10] Abnormalities of JAK/STAT pathway contribute to cellular transformation, increased cell proliferation, survival, angiogenesis and immune system evasion. Jennifer Rubin Grandis et al in 1999 and Raja R Seethala et al in 2008 showed that there is constitutive overexpression and activation of STAT3 in OSCC compared to normal mucosa. Antiapoptotic protein BCL-2 is located on chromosome 18q21, controls integrity of outer mitochondrial membrane. Regulation of apoptosis by modulating cell death regulatory protein is an integral function of oncogenesis.Stat3 regulates transcription of bcl-2 gene most likely through multiple stat3 binding elements found within bcl-2 promoter region.[11-14]

In our study bcl-2 cytoplasmic positivity was found in 23.3% of OSCC and 6.6% of VC which is in concordance with studies done by LMuzio et al (2003) , BrankaPopović et al (2007) and Drachenberg et al (1997).[15,16] The number of cells expressing bcl-2 increased from well differentiated to poorly differentiated, hence showing an inverse relation with degree of differentiation which is in concordance with studies done by Yu chen et al (2000), Singh et al (1995), Muzio et al (2003), Reshmi G Nair et al (2011) and Suri C et al (2009).[17-19]

This observation of an increase in the number of bcl-2 positive cells with a decrease in the differentiation probably reflects that bcl-2 is expressed in keratinocytes that have an increased capacity for survival. Hence, bcl-2 can be considered as an adverse prognostic marker in the evaluation of OSCC. bcl-2 was expressed in basal cells of normal mucosa and verrucous hyperplasia which is in concordance with studies by Hockenbery et al in 1991, L Lo Muzio et al in 2003 and Singh et al in 1997

indicating that it is involved in preservation of an adequate reservoir of proliferating stem cells.[20] Statistically significant association between pJak2 and stat3 immunoexpression was observed in all the three groups (I, II and III). Jak-Stat pathway was operable in 66.6% of OSCC cases (both with lymph node and without lymph node metastasis) and in 23% of Verrucous carcinoma, which is in concordance with studies done by Bowman et al., 2000 and H Sivash et al., 2004, showing ability of JAK family of kinases to drive the neoplastic transformation by STAT proteins in the development of OSCC.[21,22]

13.3% cases were positive for stat3 and negative for pJak2 indicating constitutive activation of stat by alternative pathways which was in concordance with study done by Sriuranpong et al., 2003, showing that stat can also be activated by other pathways as well such as Src, epidermal growth factor receptor, Bcr-abl, alpha7 nicotinic receptor and interleukin receptor.[23]

Statistically significant correlation was observed between stat3 and bcl-2 immunoexpression in OSCC without lymph node metastasis (Group I) which is in concordance with studies done by Shah N G et al., 2009; Jl Song et al and JrGrandis et al., 2000; Bromberg et al., 1999 and Zushi et al., 1998; showing thattargeting stat3 causes down-modulation of bcl-2 in OSCC.[24-26]

pJak2 and stat3 were not expressed in normal mucosa but expressed strongly in OSCC (Group I+II), which was in concordance with the studies done by Zhenbing et al, (2011) and Jinbo Yang et al, (2005); suggesting that overexpression of these biomarkers are associated with malignant transformation.[27]

Several approaches have been used to inhibit STAT3 in the hopes of developing an antitumor agent. Although several STAT3-specific agents are promising, none are in clinical development, mostly because of drug delivery and stability issues. In contrast, several JAK inhibitors are in clinical development. These orally available, ATP-competitive, small-molecule kinase inhibitors are being tested and future studies will determine whether JAK inhibitors are useful in the treatment of OSCC.

CONCLUSION

Oral cancer is the third most common in India with most common age of presentation being 4th-5th decade. Immunoexpression of bcl-2 revealed a bad prognostic marker as it showed inverse correlation with tumor differentiation in OSCC. Majority of these tumors show involvement of JAK-STAT pathway in the tumor development and progression which accounts for 66.6% in OSCC and 23% in VC. Further clinical trials and molecularly targeted research on JAK-P, STAT3 and BCL-2 is required to improve the treatment, patient care and prognosis as it is the major pathway involved in development of OSCC.

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