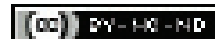


Expression of p16 and Galectin-3 in Squamous Cell Carcinoma of Uterine Cervix, in Relation to the Histomorphological Variants: A Cross-sectional Analysis

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ABSTRACT

Introduction: Cancer of uterine cervix comprises a big chunk of cancer registration worldwide. Now-a-days the immunohistochemical marker p16 has emerged as the surrogate marker of high risk Human Papilloma Virus (HPV) infection in cervical tissue. Galectin-3, a ubiquitous agent likely to modulate different pro-survival properties necessary for neoplastic cells, is recently emerging as the guardian of tumour microenvironment.

Aim: To study the expression of p16 and galectin-3 in different histomorphological variants of cervical Squamous Cell Carcinoma (SCC) and their association with grade and stage.

Materials and Methods: An observational cross-sectional study was undertaken in the Department of Pathology in a tertiary care hospital in East India, from January 2019 to June 2020. Fifty three samples diagnosed as invasive Squamous Cell Carcinoma (SCC) of uterine cervix were taken by systematic random sampling. Immunohistochemical examination was done using monoclonal antibodies against p16 and galectin-3 after obtaining thin sections from formalin fixed paraffin embedded blocks and retrieval of antigen. The data was interpreted by light microscopy using a semi-quantitative method with respect to prefixed parameters and statistical analysis was done by Chi-square test and Fisher's exact test using Statistical Package for the Social Science (SPSS) version 25.0.

Results: Fifty two out of fifty three cases (98.1%) of squamous cell carcinoma in this study showed almost 100% block positivity of p16 in the tumour cells -strongly corroborative with high risk HPV infection. The non-keratinizing and the basaloid variant showed the strongest intensity of staining (3+). Only one case showed complete negativity of p16 expression. In galectin-3 positive cases, strong expression of this marker is found in the invasive tongues of the tumour cells at the junction of tumour stromal interface, consistent with our knowledge regarding the importance of galectin-3 in regulating the tumour microenvironment. The strongest galectin-3 positivity(3+) was found in the single case of Lymphoepithelioma like squamous cell carcinoma and showed almost 100% positivity among the neoplastic cell population; whereas the non-keratinizing and Basaloid variant showed almost negative expression. Significant association ($p=0.00021$) found between tumour grade and p16 intensity.

Conclusion: The non-keratinizing and basaloid variants of squamous cell carcinoma have shown statistically significant association with highest intensity of p16 staining along with diminished expression of galectin-3. Increased tumour grade is also significantly associated with strong staining intensity of p16 and decreased galectin-3 expression. However, no significant association is found between galectin-3 expression or intensity of p16 expression and the stage of tumour.

Keywords: Basaloid, Non-keratinizing squamous cell carcinoma, Tumour micro-environment

INTRODUCTION

Cancer of uterine cervix is the second or third most common cancer in women with approximately 0.5 million cases worldwide [1]. Total 76% of recent cases occur in low resource nations [1] and in developing countries like India.

The recognition of cervical premalignant conditions, their association with high risk human papilloma virus subtypes have made amenable the disease for screening and prevention. The introduction of Papanicolaou (PAP) smear screening in the women of reproductive age group as well as routine vaccination against high risk HPVs in adolescent girls have in fact, revolutionised the approach of managing this killer disease in western countries. However, lack of these public health measures in our country to the population who deserve it most; leads to the diagnosis of Carcinoma (ca) cervix in florid invasive stage most often; and so even in this era, it appears to be a major community health problem in the non-communicable chronic disease category in this part of the world [1].

Among the different morphological variants, squamous cell carcinoma is the most common type of cervical cancer [1] and is well known to evolve through definite premalignant conditions known as cervical intra-epithelial neoplasms or squamous in-situ lesions. There are

eight recognized histomorphological variants of cervical SCC namely 1. Keratinizing 2. Non-Keratinizing 3. Basaloid 4. Warty 5. Papillary 6. Verrucous 7. Squamotransitional 8. Lymphoepithelioma Like [1]. The role of high risk HPV infections (HPV 16 and 18) among atleast 70 genetically distinct type of HPVs [2], the pathogenesis of SCC cervix is now well established [3].

So far the the pathogenesis of ca cervix is known to progress after integration of high risk HPV genome to the host genome, leading to interruption of viral Deoxyribonucleic Acid (DNA) within E1/E2 reading frame, loss of E2 viral repressor and increased expression of E6 and E7 oncoproteins [4]. The E6 protein binds and mediates the degradation of p53 [5] and stimulates the expression of Telomerase Reverse Transcriptase (TERT). E7 binds to Retinoblastoma (RB) protein in the active hypophosphorylated form which normally sequesters transcription factor E2F in the same binding site, thus displaces it and drives the progression of cell cycle crossing the G1-S check point. As a result there is compensatory over expression of upstream regulators like p16 [5-9], which inactivate cyclinD-CDK4 and cyclin D-CDK6 complexes trying to retain RB in active hypophosphorylated form.

Thus, persistent infection by high risk HPVs leads confluent nuclear and cytoplasmic positivity of p16 in cells;- otherwise known as

block positivity. As the detection of HPVs by hybridization or other molecular methods is quite cumbersome and pretty costly; p16 positivity in cervix gradually emerged as the surrogate marker of high risk HPV infection [10,11].

Galectins are carbohydrate binding proteins having high affinity for beta galactosides. Galectin-3 is the lone member of the chimeric group having a single Carbohydrate Recognition Domain (CRD) with a unique N-terminal domain. The Novel Antiapoptotic Molecule with functional BH1 (NWGR) motif within CRD is important for interactions with various anti-apoptotic B-cell Lymphoma/Leukaemia-2 (BCL2) family proteins [12]. Also it has the unique ability to form pentamers and it allows to form lattices with glycolipids and glycoproteins [12].

Recent studies are focussing on the tumour microenvironment more often after realisation of the fact that the cells of tumour niche may be drivers of tumourogenesis rather than acting as mere bystanders or supportive cells. In this scenario, galectin-3, a ubiquitous agent likely to modulate different pro-survival properties necessary for neoplastic cells like 1) positively regulating survival signalling [13] and suppressing stress pathways [14], 2) blocking immune surveillance [15] by inhibition of immune cells [16], 3) modulating cell adhesion [17,18] to regulate contact with stromal cells, promoting metastasis and skewing tumour cells homing to protective niches 4) suppressing tumor cell differentiation 5) controlling endocytosis of critical cell surface receptors 6) regulating cell survival cascades essential for tumour cells to survive changes in oxygen and metabolite content [19-21], is recently emerging as the guardian of tumour microenvironment.

The role of galectin-3 in various cancers is complex [22-29]. The elevated levels have been shown to prognosticate for poor survival in cancers like lymphoma [30], leukaemia [31], breast cancer and thyroid cancer [32] but decreased level appears to be detrimental to patients suffering from chronic lymphoblastic leukaemia and prostate cancer. A possible explanation can involve the intracellular location of the marker [33].

Although association of positive p16 expression with high risk HPV related cases of squamous cell carcinoma of uterine cervix is now a well established fact, there are instances of p16 negative cases also [34]. As p16 is considered as the surrogate marker of HPV infection, this study is expected to re-evaluate its role in progression of cervical SCC, especially the relative contribution of HPV in different morphological variants.

Owing to the emerging role of galectin-3 as the guardian of tumour microenvironment, there are conflicting results [35-43] on its role in cervical tumour biology from different studies, some have documented a positive correlation between its expression and increased tumour invasion [36-41]; while some have demonstrated the opposite [35,37]. Also its differential expression in different morphological variants of SCC has not been studied before.

Objectives

1. To study the expression of p16 and galectin-3 in different histomorphological variants of cervical squamous cell carcinoma
2. To study the association of the expression profile of those two markers with tumour grade and stage

MATERIALS AND METHODS

An observational, descriptive, cross-sectional study was undertaken in the Department of Pathology in a tertiary care Institution of Kolkata from January 2019 to June 2020 (IEC reg no: ECR/322/Inst/WB/2013). Fifty three samples diagnosed as invasive SCC of uterine cervix were taken by systematic random sampling.

Inclusion criteria: Histopathological specimens received in the Department of Pathology within the study period (either received as cervical punch biopsy or surgically excised specimen) morphologically diagnosed as squamous cell carcinoma of uterine cervix were included.

Exclusion criteria: The specimens of benign cervical lesions, cervical intraepithelial neoplasms or squamous in-situ lesions, or of cervical carcinoma morphologically different from squamous cell carcinoma like cervical adenocarcinoma were excluded from the study.

Ultrathin (3-4 microns) sections are obtained by microtomy from the formalin fixed paraffin embedded blocks. After floatation they were picked on poly-L-lysine coated slides, dried, deparaffinized and rehydrated in descending grades of alcohol.

Heat Induced Epitope Retrieval (HIER) procedure was done by microwave method using Tris Hydroxymethyl Aminomethane (TRIS) Buffer, EMPARTA, pH 9.0. TRIS Buffer (EMPARTA, pH 7.2) was used for washing. Endogenous peroxidase activity was blocked with PolyExcel Peroxidase Block, (PATHNSITU) Incubation with primary antibody (Monoclonal antibody against p16-p16 G175405 MonC, PATHNSITU & Monoclonal antibody against galectin-3-Galectin-3-9 MIB, PATHNSITU) was done at 37°C for 60 minutes. For visualisation of result, serial incubation for 30 minutes each was carried out with PolyExcel Target Binder, PATHNSITU; Poly Horse Radish Peroxidase (HRP) (PolyExcel HRP DAB Detection System, PATHNSITU) and chromogen (Polyexcel Stunn DAB Buffer & Polyexcel Stunn Diaminobenzidine (DAB) Chromogen, PATHNSITU).

The sections were then counterstained with Harris Haematoxylin and mounted. Sections of chronic cervicitis were taken as control group. For validation of galectin-3 staining; section of Papillary Thyroid carcinoma was used as positive control.

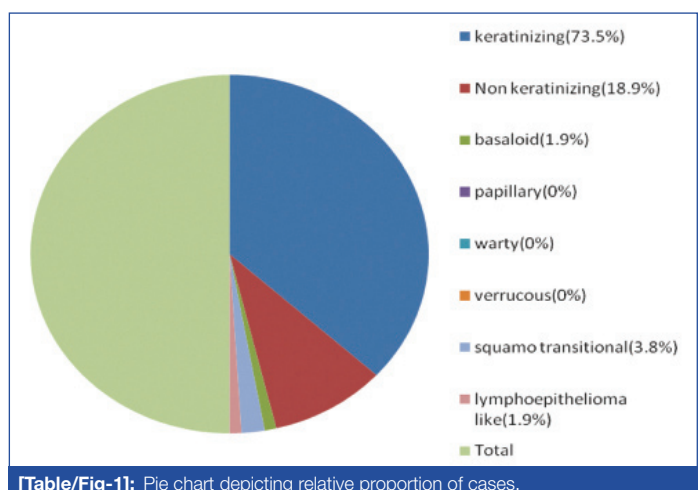
Proportional average expression of p16 and galectin-3 were allocated by semi quantitative method using light microscopy, based on the overall impression, after scrutinizing the whole slide especially focussing on the hot spot zones. Intensity of the immunostaining were taken as 1+, 2+, 3+ depending upon the positivity. For statistical purposes, 75% proportional positivity of p16 and 50% positivity of galectin-3 in the neoplastic cell population were taken as positive [38].

STATISTICAL ANALYSIS

The data was collected and analysed by Chi-square test and Fisher's exact test using SPSS, version 25.0.

RESULTS

Total of 73.5% (39/53) cases are Keratinizing variant of squamous cell carcinoma (most prevalent type in this study) succeeded by Non-keratinizing variant (10/53) (18.9%). Two cases of squamo transitional (3.8%) and one case each from the Basaloid (1.9%) and Lymphoepithelioma (1.9%) like variant were included in the study. No cases of Papillary, verrucous and warty variants were found within the proposed study duration, so these three variants are not included [Table/Fig-1].



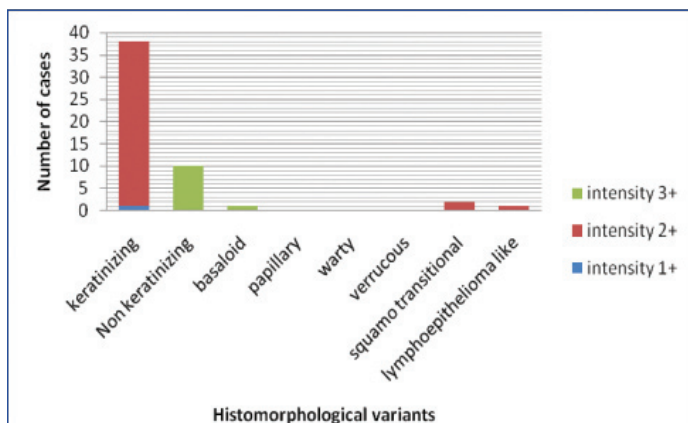
[Table/Fig-1]: Pie chart depicting relative proportion of cases.

All but one case (which belongs to the keratinizing variant) showed positive expression of p16 [Table/Fig-2].

Histological type	P16 expression		Total
	Positive	Negative	
Keratinizing	38 (97.4%)	1 (2.6%)	39 (100%)
Non keratinizing	10 (100%)	0 (0%)	10 (100%)
Basaloid	1 (100%)	0 (0%)	1 (100%)
Squamo transitional	2 (100%)	0 (0%)	2 (100%)
Lymphoepithelioma like	1 (100%)	0 (0%)	1 (100%)
Total	52 (98.1%)	1 (1.9%)	53 (100%)

[Table/Fig-2]: Distribution of p16 positivity in different variants of cervical squamous cell carcinoma (n=53).

100% of Non-keratinizing and Basaloid variant showed highest (3+) intensity of p16 expression and 97.4% (37/38) of keratinizing variant showed moderate (2+) intensity of p16 staining [Table/Fig-3].



[Table/Fig-3]: Bar diagram showing distribution of p16 intensity with respect to different histomorphological variants in p16 positive cases.

Galectin was positive in keratinizing, non-keratinizing, squamo transitional and lymphoepithelioma variant. However it showed 100% negativity in basaloid variant [Table/Fig-4].

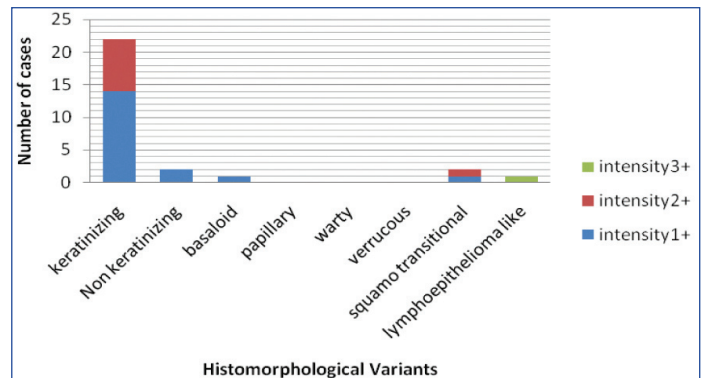
Histological type	Galectin-3 status		Total
	Positive	Negative	
Keratinizing	21 (53.8%)	18 (46.2%)	39 (100%)
Non keratinizing	1 (10%)	9 (90%)	10 (100%)
Basaloid	0 (0%)	1 (100%)	1 (100%)
Squamo transitional	2 (100%)	0 (0%)	2 (100%)
Lymphoepithelioma like	1 (100%)	0 (0%)	1 (100%)
Total	25 (47.2%)	28 (52.8%)	53 (100%)

[Table/Fig-4]: Distribution of Galectin-3 status among different variants of cervical squamous cell carcinoma (n=53).

The single case of Lymphoepithelioma like variant showed strongest (3+) intensity of galectin-3 expression. Those cases of non-keratinizing and basaloid variant which were positive for Galectin-3, only showed weak (1+) expression. In 63.6% (14/22) of the cases of keratinizing variant showing galectin-3 positivity, showed 1+intensity; the rest showed moderate (2+) intensity of staining. Among the two cases of squamo-transitional variant, one showed 1+and the other showed 2+intensity of staining [Table/Fig-5].

Significant association was found between tumour grade and p16 intensity in p16 positive cases. ($p=0.0002111$) [Table/Fig-6].

Proportion of galectin-3 positive cases among grade 1, grade 2 and grade 3 tumours are 66.7%, 71.9% and 16.7% respectively [Table/Fig-7]. Statistically significant association was found between galectin-3 positivity and the different histomorphological variants of cervical squamous cell carcinoma ($p=0.00448$). Although no significant association was found between the positivity

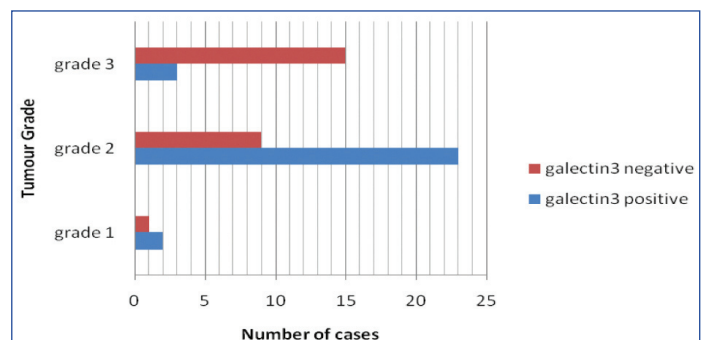


[Table/Fig-5]: Bar Diagram showing Distribution of intensity of galectin-3 among different variants of galectin-3 positive squamous cell carcinomas.

Grade	p16 staining Intensity			Total
	1+	2+	3+	
Grade 1	1 (33.3%)	2 (66.7%)	0 (0%)	3 (100%)
Grade 2	0 (0%)	29 (93.5%)	2 (6.5%)	31 (100%)
Grade 3	0 (0%)	9 (50%)	9 (50%)	18 (100%)
Total	1 (1.9%)	40 (76.9%)	11 (21.2%)	52 (100%)

[Table/Fig-6]: Distribution of cases according to Tumour grade and p16 intensity in p16 positive cases (n=52).

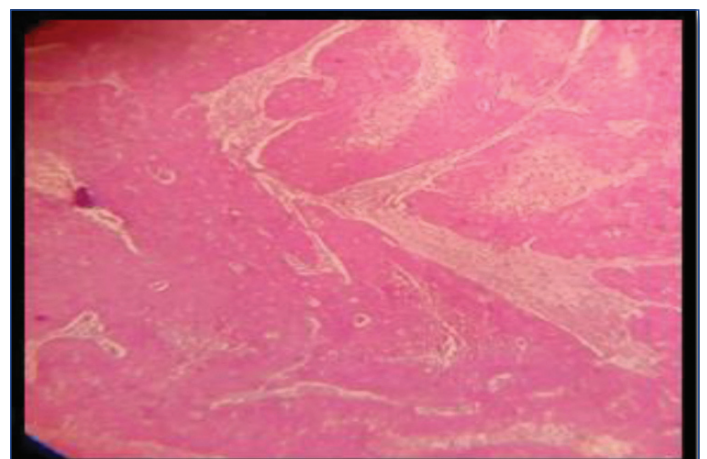
($p=0.857344$) or intensity of galectin-3 ($p=0.211128$) and Tumour stage, it becomes apparent that galectin-3 positivity decreases with increasing grade ($p=0.000771$) [Table/Fig-7].



[Table/Fig-7]: Bar diagram showing Distribution of cases according to Tumour grade and Galectin 3 status.

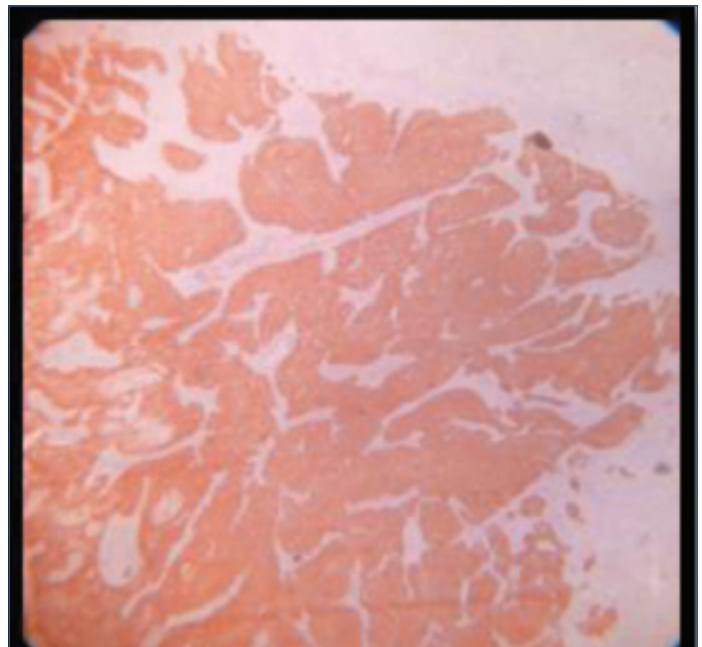
DISCUSSION

Among the cases of SCC cervix dealt in the present study, belonging to a wide age range of 32 years to 78 years, Keratinizing subtype [Table/Fig-8] is the predominant variant encountered among all the age groups succeeded by Non Keratinizing subtype [Table/Fig-1]. Majority of these cases (83.0%) were diagnosed in an advanced stage (clinically International Federation of Gynaecology and Obstetrics (FIGO) stage II B or greater, that is cancer with parametrial invasion or higher) and hence rendered as inoperable.

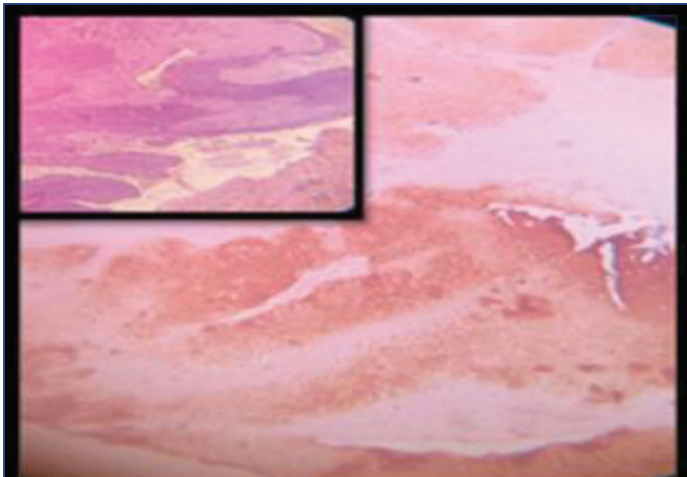


[Table/Fig-8]: Keratinizing SCC, (H&E, 100x).

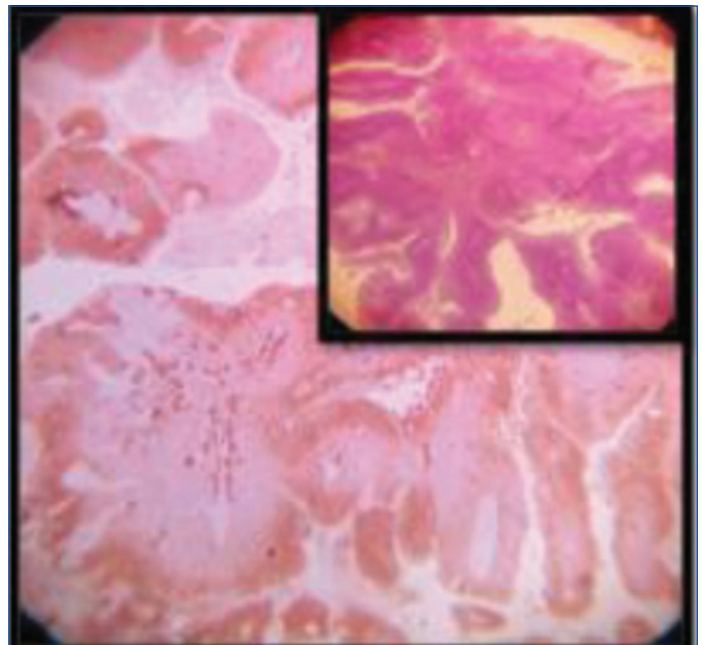
All but one of the 53 cases in this study showed almost 100% block positivity of p16 in the tumour cells -strongly corroborative with high risk HPV infection [Table/Fig-2] but the intensity of staining is variable among different variants. The non-keratinizing and the basaloid variant (100%) showed the strongest intensity of staining (3+) in the present study [Table/Fig-3,9,10], which is consistent with the researches carried out Internationally; focussing on specific histomorphological variants of squamous cell carcinoma, those are most consistently associated with high risk HPV infection [1,42]. Total 97.4% of the Keratinizing variant showed intermediate positivity (2+) [Table/Fig-11]. And also the more differentiated it becomes, less becomes the intensity of p16 staining. Even in the same tumour, the foci of relatively poorly differentiated areas showed more intense staining. And this association between the intensity of staining of p16 with the histomorphological variants of Squamous cell carcinoma in cases showing positive p16 expression is statistically significant ($p < 0.0001$). The squamo columnar junction or the transition zone invariably showed block positivity of p16, which supports our present knowledge regarding the predilection of high risk HPVs towards this region. The squamotransitional variant has also shown 2+intensity of p16 expression [Table/Fig-12].



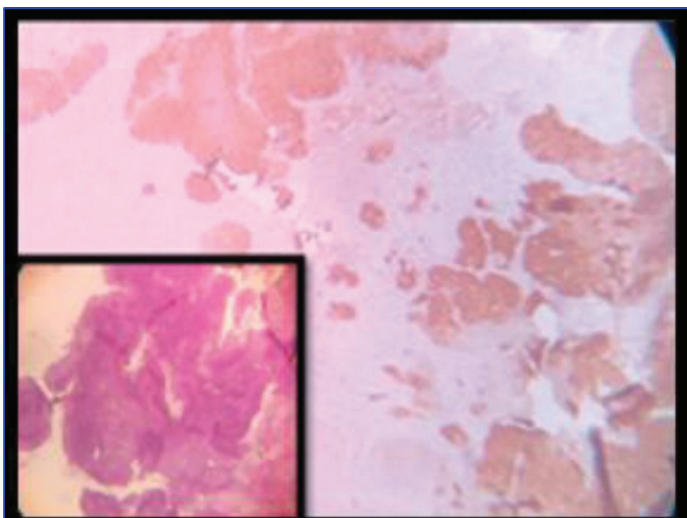
[Table/Fig-11]: p16 expression (2+) in keratinizing SCC (4x).



[Table/Fig-9]: Non Keratinizing SCC: p16 expression(3+); Inset : H&E of the same (100x).



[Table/Fig-12]: Squamotransitional SCC: p16 expression(2+); Inset : H&E of the same (100x).



[Table/Fig-10]: Basaloid SCC: p16 expression(3+); Inset : H&E of the same (100x).

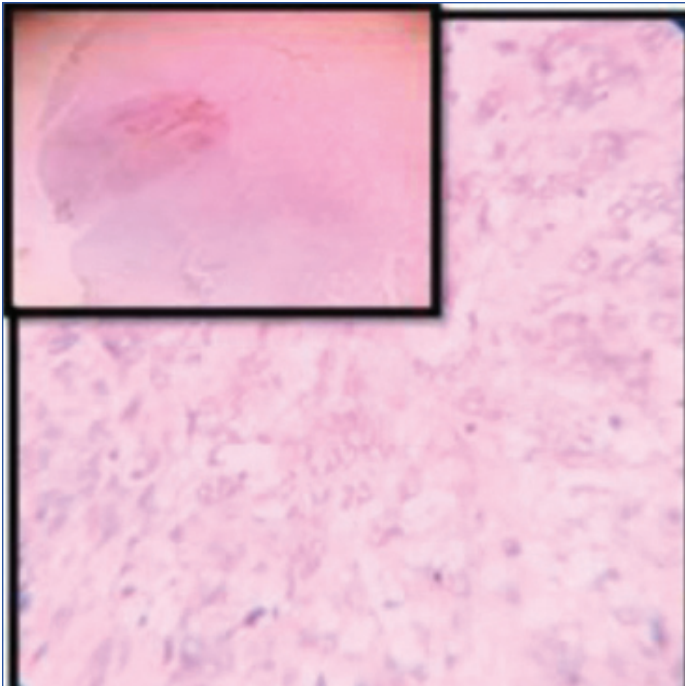
Nuclear grade in case of cervical SCC does not carry much prognostic significance [43]. Same thing can be said for the different histomorphological variants once the clinical stages are matched [43]. However, it is apparent from the present study that Intensity of p16 staining increases with increasing grade ($p = 0.0002$) [Table/Fig-6], which is consistent with our existing knowledge of predilection of high risk HPVs as the causative agent of relatively undifferentiated forms of cervical squamous cell carcinoma [1].

However, no significant association was found between tumour stage and intensity of p16 expression.

Though not very common, there are instances of p16 negative squamous cell carcinomas in literature. The verrucous variant usually shows p16 negativity. Here authors have found a completely p16 negative case of keratinizing squamous cell carcinoma of uterine cervix [Table/Fig-13]. (The authenticity of this finding is proven by the p16 positivity of the squamocolumnar junction in the same slide, which serves as an internal positive control).

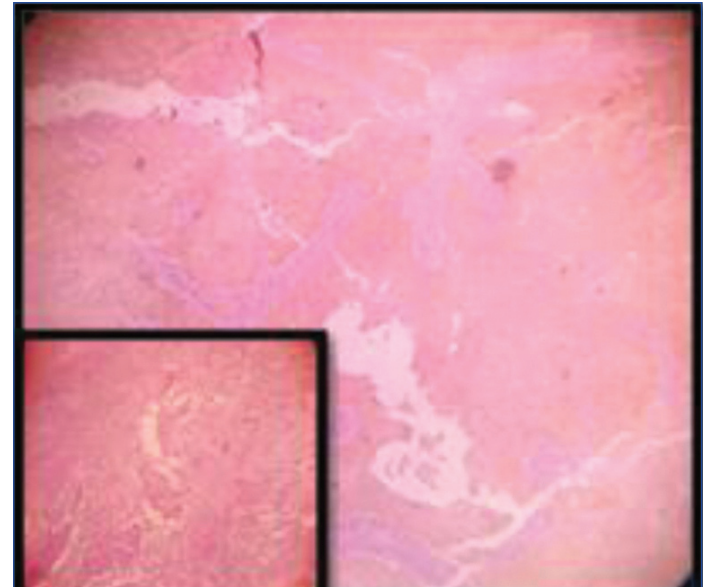
In this case, The p16 positivity of the squamo-columnar junction indicates towards persistent infection of high risk HPV and presumably it was also a key event behind the carcinogenesis as it is commonly seen in other cases. Probably in a late stage of carcinogenesis, a p16 mutation was acquired by the neoplastic clone and therefore the picture we get is that of complete negativity of p16 expression. Similar case of p16 negative squamous cell carcinoma of cervix is reported by the study conducted by Gupta R et al [44] and Volgareva G et al [45].

Regarding the expression of galectin-3, mostly the tumour cells showed cytoplasmic localisation of this marker in the cases of SCC



[Table/Fig-13]: p16 negative cervical SCC (H&E:40 X); inset (H&E:4X): squamocolumnar junction.

from the study of Punt S et al [37]. The squamotransitional variant has also shown 2+intensity of galectin-3 expression [Table/Fig-16].

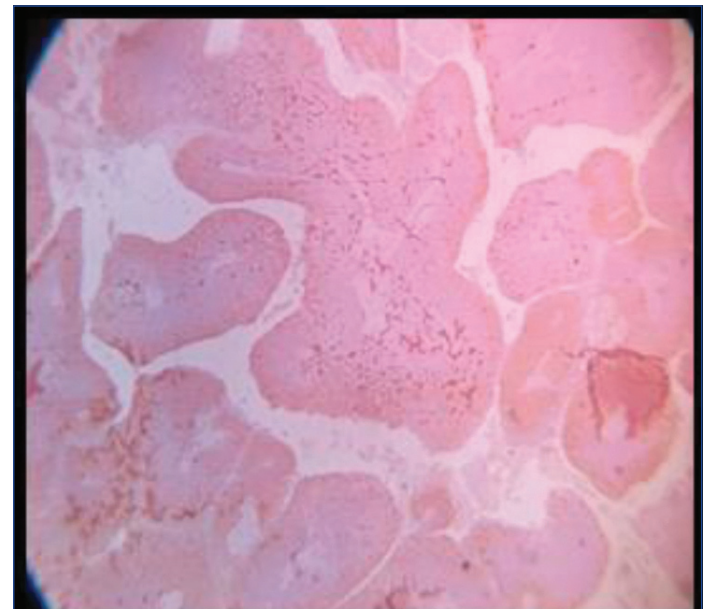


[Table/Fig-15]: Lymphoepithelioma like SCC: galectin-3 expression(3+) (10x); Inset: H&E of the same (10x).

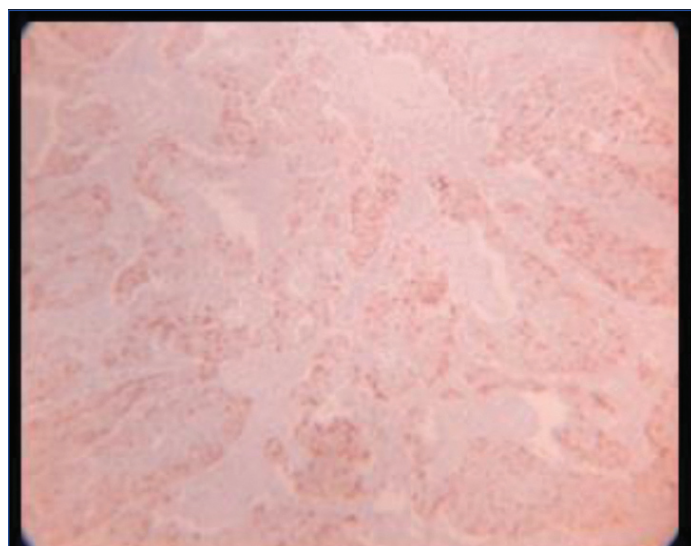
cervix dealt in this study. The interesting thing is that in galectin-3 positive cases, strong expression of this marker is found in the invasive tongues of the tumour cells at the junction of tumour stromal interface which is consistent with our knowledge of importance of galectin-3 in regulating the tumour microenvironment. This finding is also consistent with the findings described by Punt S et al., [37].

In contrary to the findings of Lee JW et al [35], Kim SS et al., [38] concluded that both the positivity of intensity of galectin-3 expression increased as the lesion progresses from intra epithelial lesions to invasive cancer. However, Punt S et al., found that weak and positive galectin-3 expression in tumour cells were correlated with increased and decreased tumour invasion respectively [37].

Based on the 50% cut off criteria, 47.2% of total cases were positive for galectin-3 expression. Mostly the keratinized variants (53.8%) showed positivity of galectin-3 [Table/Fig-14] whereas the non-keratinizing and Basaloid variant showed almost negative expression (90% of cases) [Table/Fig-4]. The strongest galectin-3 positivity (3+) was found in the single case of Lymphoepithelioma like squamous cell carcinoma and showed almost 100% positivity among the neoplastic cell population [Table/Fig-5,15]. And thus this usually suggests tumour galectin-3 expression may be necessary for a differentiated phenotype; which again corroborates with the findings



[Table/Fig-16]: Squamotransitional SCC: galectin-3 expression(2+) (10x).



[Table/Fig-14]: Galectin-3 expression(2+) in Keratinizing SCC (4x).

Over all it comes forth that the more poorly differentiated variants like Non-Keratinizing and Basaloid subtype have a propensity to show strong expression of p16 and negative or weak expression of Galectin-3.

Probably it will be best to conclude as it was inferred from the study of Punt S et al., [37] - owing to the diverse and complex actions of galectin-3 in regulating almost all the hallmarks of cancer, it is difficult to draw any direct correlation regarding its expression and the behaviour of invasive cervical cancer. So, in this scenario, it will be challenging to use this marker as a tool of diagnostic or prognostic utility.

Limitation(s)

No case of Papillary, Verrucous and warty variant of cervical squamous cell carcinoma was found within the stipulated time period. So, no inference can be drawn regarding the behaviour of those histomorphological variants from this study.

CONCLUSION(S)

The basaloid and non-keratinizing variant of squamous cell carcinoma show highest intensity of p16 staining consistent and

is associated with high risk HPV infection. Though, most of the cases of cervical squamous cell carcinoma show block positivity of p16 and are associated with high risk HPV infection; cases of p16 negative cervical SCC also exist. Statistically significant association is found between histomorphological variant of SCC cervix and galectin-3 expression as well as tumour grade and galectin-3 positivity with decrease in staining in relation to higher grade; consistent with the assumption that galectin-3 positivity is necessary for a differentiated phenotype. However, the association between tumour stage and expression of this marker was not significant in the present study.

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