Expression of p16CDKN2A On Urothelial Carcinoma Of Bladder And Its Relationship With Grading And Staging: A Clinico-Histopathological Correlation

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ABSTRACT

Background of present study: p16 is an inhibitor of cyclin dependent kinases, which selectively inhibits Cyclin D and CDK4 and thereby it slows down the passage of cell at the time of crossing G1-S check point and hence acts as a tumour suppressor. Urothelial carcinoma, invasive and non invasive are heterogeneous in morphology, clinical behaviour and prognosis. Dysregulation of p53 related pathway are common in high grade papillary urothelial carcinoma and invasive urothelial carcinoma. p16 CDKN2A, which acts as an upstream regulator of cell-cycle pathway and plays important role in pathogenesis of several cancers, is emerging as an important factor behind pathogenesis of urothelial carcinoma.

Objective of proposed research:

i) To estimate the expression of p16 in different variants of urothelial carcinoma
ii) To determine the grading and staging of carcinoma from TURBT chips
iii) To determine the association using appropriate statistical software.

Methodology: An observational cross sectional study was undertaken in the department of pathology in a tertiary care hospital in Eastern India, of 6 months duration. 28 samples diagnosed as Urothelial carcinoma of bladder were taken by systematic random sampling as per the inclusion-exclusion criteria from the TURBT specimens and immunohistochemical examination was done using monoclonal antibodies against p16.

Outcome: Overall, 42.8% cases were found to be p16 positive in our study based on the pre fixed parameter. 84.6% of the low grade cancers showed positive expression of this marker, where as only 6.6% of the high grade cancers were positive. Higher proportion of p16 expression is found in the non-invasive urothelial carcinomas (83.3% as compared to 12.5% in invasive variety). Decreased expression of p16 corroborates with increased Tumour invasion and aggressiveness and this finding is statistically significant.

Conclusion: Low grade and Non-invasive types of Urothelial carcinoma showed propensity towards positive expression of p16. Statistically significant association is found with negativity of expression of this marker with higher stage and deep muscle invasion; suggestive of silencing of p16 tumour suppressor with increased aggressiveness as well as Tumour grade.

Keywords: Urothelial Neoplasm, P16, Expression, Immunohistochemistry
INTRODUCTION:
Carcinoma of urinary bladder contributes to about 7% of estimated cancer incidence in male and accounts for at least 4% of estimated cancer related deaths (as per American cancer society, cancer statistics).[1] Although infectious agents like Schistosoma hematobium plays an important role in endemic areas like Egypt and Sudan, especially in the emergence of squamous cell carcinoma of bladder, historically epidemiological data has always drawn our attention with its particular relationship with chemical carcinogens. Cigarette smoking, like its better known association with lung cancer is also a common aetiological agent here. Which is worth mentioning, bladder tumours are closely associated with industrialisation (particularly petrochemical industry), clustering of cases show strong urban predilection with its well-known association with exposure of aryl amines or aniline dyes (particularly benzidine and 2-napthylamine). It emphasizes the role of addressing this issue in the domain of industrial health and ergonomics.[1]

Haematuria is the commonest presenting symptom in the vast majority of patients. Though urine is considered as the liquid biopsy of urinary tract, urine cytology is not an efficient tool for screening in case of urothelial neoplasm, as it is the case of Pap smear in the domain of carcinoma of uterine cervix. Usually Trans urethral resection of bladder tumour (TURBT) with sampling of deep muscle is done with the aid of cystoscopy, for diagnosis and staging. Conservative management like Intra vesical chemotherapy, Immunotherapy with BCG is commonly carried out; Radical cystectomy with ileal conduit is undertaken only in cases with higher stages showing extensive deep muscle invasion.

BACKGROUND OF RESEARCH:
Malignant Tumours of urothelial differentiation is the most important subtype encountered in daily practice while evaluating the carcinoma of bladder though; other subtypes like squamous cell carcinoma, adenocarcinoma arising from urachal remnants and neuroendocrine tumours are also found occasionally. Broadly these neoplasms are categorized into two classes: invasive and non invasive based on tumour invasion of underlying lamina propria.

Invasive urothelial carcinoma are characterized by their divergent mode of differentiation, squamous, glandular, and trophoblastic and usually are high grade in nature.[2] Several different histomorphological variants are well recognized, which are important for prognostication also, like: Nested, Microcystic, Micropapillary, Plasmacytoid, Lymphoepithelioma like, Giant cell, Lipid rich, Clear cell, Sarcomatoid and Poorly differentiated (including with osteoclast like giant cell).[3]

As per the classification designed by ISUP (International society of Urothelial Pathology), Non-invasive urothelial carcinomas can be flat (carcinoma in situ and by definition high grade) or papillary; which again is subdivided into low grade and high grade based on architectural and cytological characters; though entities like urothelial papilloma, inverted urothelial papilloma, papillary urothelial neoplasm of low malignant potential(PUNLUMP), urothelial dysplasia, urothelial proliferation of uncertain malignant potential are also recognised.

Papillary urothelial carcinoma is differentiated from PUNLUMP by distinguishing the loss of polarity of the constituent cells. More disordered architecture, prominent fusion of papillae, presence of solid area stand in favour of the diagnosis of a high grade tumour. [3]Cytological atypia like nuclear pleomorphism, irregular nuclear membrane, prominent nucleoli and abundant mitotic figures (often atypical one) are also some of the characteristic findings.

Pathogenesis:
Resident urothelial stem cells are transformed into cancer stem cells by acquisition of mutations and clonal expansion; it is the recent postulated hypothesis behind pathogenesis of papillary urothelial carcinoma.[3] Low grade tumours are associated with derangements of FGFR3 growth factor receptor pathway, where as high grade tumours sustain dysregulatory changes in the p53 related pathway.[4] Apart from this, the commonest mutation in non-invasive urothelial carcinoma is TERT (telomerase
reverse transcriptase catalytic subunit) promoter mutation.[4] Inactivation mutation in the cohesion complex STAG2, and the role of epigenetic silencing and role of micro RNAs are emerging as the key player behind carcinogenesis now-a-days.

Large chromosomal alterations with loss of tumour suppressors FHIT, PTCH and gain of function mutations in ERBB2, CCND1, MDM2 are commonly encountered in invasive urothelial cancers.[3] Genomic instability is quite a characteristic finding here. Derangement of FGFR3 and P53 pathway together with TERT are also the three common pathways recognized here.[4] FGFR3/RAS/RAF signalling, PI3K/Akt/m-TOR pathway, Notch receptor signalling, p53/ Rb pathway and chromatin remodelling pathway are the noteworthy signalling cascades which play important role in genesis of invasive cancer and can be therapeutically targeted.[3] p16 is a 16 kilo Dalton protein composed of 148 amino acids with four ankyrin repeats. It is encoded by the CDKN2A gene located on chromosome 9(9p21.3). The CDKN2A locus also generates ARF protein by alternative splicing of mRNA; which acts as a stabilizer of tumour suppressor p53 by interacting and sequestering MDM2.[2]

p16 is an inhibitor of cyclin dependent kinases, which selectively inhibits cyclin D and CDK 4 complex - which in the other hand are responsible for Phosphorylation of the Rb protein.[2] There by p16 slows down the passage of cell at the time of crossing G1-S check point and hence acts as a tumour suppressor.

Pancreatic adenocarcinoma is often associated with mutations of CDKN2A gene. Homozygous deletions of p16 are frequently found is esophageal and gastric carcinoma. Germline mutations of this gene predispose to variety of skin cancers most notably melanoma. Epigenetic silencing of this locus is also emerging as a novel mechanism behind the pathogenesis of varieties of neoplasms. Especially the genetic silencing by hypermethylation of DNA in case of esophageal squamous cell carcinoma needs special mention.

The role of p16 in the domain of carcinoma of uterine cervix has widely drawn attention. Persistent infection by high risk HPVs leads to compensatory over expression of p16 in cells[5] which manifests as confluent nuclear and cytoplasmic positivity of this marker;- 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.

**METHODOLOGY:**
An observational, descriptive, cross sectional study was undertaken in the department of pathology, R G Kar medical college and hospital, Kolkata of 6 months duration. 28 samples diagnosed as urothelial carcinoma of bladder were taken by systematic random sampling as per the inclusion-exclusion criteria from the received TURBT specimens in the department.

Ultrathin sections are obtained by microtomy from the formalin fixed paraffin embedded blocks. After floatation they are 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 Buffer, EMPARTA, pH 9.0. TRIS Buffer (EMPARTA, pH 7.2) was used for washing. Endogenous peroxidise activity was blocked with PolyExcel Peroxidase Block, (PATHNSITU) Incubation with primary antibody (Monoclonal antibody against p16-p16 G175405 MonC, 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 HRP (PolyExcel HRP DAB Detection System, PATHNSITU) and chromogen ((Polyexcel Stunn DAB Buffer & Polyexcel Stunn DAB Chromogen, PATHNSITU).

The sections were then counterstained with Harris Hemtoxylin and mounted. For validation of p16 staining; section of Non Keratinizing cervical squamous cell carcinoma was used as positive control. Expression of p 16 was measured by semi quantitative method using immunohistochemistry. Intensity of the immunostaining was taken as 1+, 2+, 3+ depending upon the positivity. For statistical purposes 75% proportional positivity of was taken as positive.
RESULT AND ANALYSIS:

Table 1: Distribution of cases of Urothelial carcinoma according to Tumour grade and p16 expression

<table>
<thead>
<tr>
<th>P16Expression</th>
<th>Tumour Grade</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Grade</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High Grade</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>15</td>
</tr>
</tbody>
</table>

Chi Square value-17.2786 p value-0.000032 and so significant.

Table 2: Distribution of cases of Urothelial Carcinoma according to Presence of Invasion and p16 status

<table>
<thead>
<tr>
<th>P16 Expression</th>
<th>Invasive carcinoma</th>
<th>Non invasive carcinoma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Negative</td>
<td>14</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>12</td>
<td>28</td>
</tr>
</tbody>
</table>

Chi square value 14.0486 p value-0.000178 and so significant.

Table 3: Distribution of cases of Urothelial carcinoma according to p16 expression and tumour stage

<table>
<thead>
<tr>
<th>P16 expression</th>
<th>stage Ta</th>
<th>Stage T1</th>
<th>Stage T2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>negative</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>9</td>
<td>7</td>
<td>28</td>
</tr>
</tbody>
</table>

P value-0.0089 by Fisher’s exact test and so significant.

Fig 1: Light Microscopic Photomicrograph of High Grade Invasive Urothelial Carcinoma of Bladder.

[Haematoxylin-Eosin Stain, 100X magnification]
DISCUSSION;
Among the 28 cases chosen in our study, 13 were low grade and 15 were of high grade urothelial carcinoma. 69.3% cases of low grade cancers were non invasive Papillary urothelial carcinoma and the rest showed features of invasion. Among the high grade Tumours, 80% cases were invasive, and again 58.3% showed deep muscle invasion.
In our study, p16 expression has showed both nuclear and cytoplasmic positivity, which is the usual case found in the scenario of cervical carcinoma. Overall, 42.8% cases were found to be p16 positive in our study based on the prefixed parameter. 84.6% of the low grade cancers showed positive expression of this marker, where as only 6.6% of the high grade cancers were positive.[Table 1, Figure 1, Figure 2] And this expression pattern of this marker with tumour grade is statistically significant.
Higher proportion of p16 expression is found in the non invasive urothelial carcinomas (83.3% as compared to 12.5% in invasive variety).[Table 2] This association was found significant statistically.
Overall, it seems decreased expression of p16 corroborates with increased Tumour invasion and aggressiveness. No tumour with positive expression has shown deep muscle invasion in our study. whereas 43.7% of the tumours showing negative expression has shown deep muscle invasion and hence rendered as stage T2 cancer as per the staging based on AJCC, 8th edition.[Table 3] (p value-0.0089 and so significant).
Raspollini MR et al studied TURBT specimens of invasive and non invasive papillary urothelial carcinomas and found p16 positivity in 28.3% cases. According to them, the expression of p16 was significantly correlated with disease stage but not with grade and progression.[6]
Yang CC et al showed significant difference of p16 staining between the normal bladder mucosa and cancer tissue. The p16 expression increases with the higher clinical stage and grade and the expression is higher in the non invasive group in comparison to the invasive tumours. They also suggested that the expression of p16 is inversely proportional to the expression of cyclin D1 and this pattern is consistent in urothelial carcinoma.[7]
Xiaoning Gan et al performed a meta-analysis based on 37 studies and 2246 cases regarding the expression of this marker in urothelial carcinoma. Down regulated p16 expression, according to them, is an index of poor prognosis, considering in terms of recurrence free, progression free and over all survival. They also demonstrated an association between low expression of p16 with clinical stage and lymph node metastasis.[8]
Ching-Hsiu Yang et al analysed expression of p16 and p27 on tissue microarrays constructed from paraffin embedded specimens. High p16 invasion, according to them was associated with absent tumour invasion and low grade carcinoma. They concluded the expression of this marker carries prognostic significance and can be exploited as a therapeutic target.[9]
Stefan Krüger et al found significant correlation between loss of p16 expression and tumour progression in minimally invasive bladder carcinomas. Loss of p16 was found in 54% of the cases in their study.[10]
Atif Ali Hashmi et al performed p16 immunohistochemistry on 161 samples and found low expression in 86% cases. In contray to the previous studies, low grade tumours showed less intensity of staining compared to their high grade counterparts and invasive tumours with lamina propria or
musculaispropria invasion were associated with higher p16 expression. In addition to this, high p16 expression was associated with decreased overall survival- was concluded by them.[11]

CONCLUSION:
Low grade and Non invasive types of Urothelial carcinoma showed propensity towards positive expression of p16. Statistically significant association is found with negativity of expression of this marker with higher stage and deep muscle invasion; suggestive of silencing of p16 tumour suppressor with increased aggressiveness as well as Tumour grade. Though these findings are consistent with the result published in the international journals on similar studies, owing to the small sample size, the results need further evaluation in a wider set-up.

REFERENCES:

How To Cite This Article:

Source of Support: Nil

Conflict of Interest: None declared

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