Immunohistochemical Expression of PDL1 in Pre-Malignant and Malignant Lesions of Cervix

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ABSTRACT

BACKGROUND

Cervical cancer screening programs are active in most countries, and is more important in India because of the large incidence of cervical cancer here. Association with Human Papilloma Virus has also been well established. However, host immune response and hence role of Programmed Death Ligand receptors are being investigated extensively.

METHODS

We included 100 cases of in situ and invasive cervical cancer, with proper prior consent after obtaining institutional ethical clearance. Histopathological diagnosis, in light of clinico-radiological findings were correlated with PDL1 expression (Dako) on paraffin embedded tissue by immunohistochemistry.

RESULTS

We found that there was stronger expression of PD-L1 (membranous and cytoplasmic) in malignant cases as compared to premalignant cases and difference in score and grade among premalignant and malignant cases was found to be statistically significant.

CONCLUSIONS

This study supports the theory that expression of PD-L1 can serve as a potent mechanism for potentially immunogenic tumours to escape from host immune responses.

KEYWORDS

Cervical, Cancer, PDL1, Immune Response

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DOI: 10.18410/jebmh/2020/265

Financial or Other Competing Interests: None.

How to Cite This Article:
Nandini R, Jaiswal R, Singh N, et al. Immunohistochemical expression of pdl1 in pre-malignant and malignant lesions of cervix. J. Evid. Based Med. Healthc. 2020; 7(26), 1245-1249. DOI: 10.18410/jebmh/2020/265

Submission 03-05-2020,
Peer Review 07-05-2020,
Acceptance 08-06-2020,
Published 28-06-2020.

BACKGROUND

Cervical cancer is a major healthcare issue for women worldwide. In India, the incidence and prevalence may be kept under check by increasing hygiene awareness and level of education. Participation in screening programs and use of government approved vaccine can reduce its burden. Normal cells gradually transform to cancer cells through several stages, and changes occurring during the transformational stages need to be assessed involving a series of epidemiologic, clinicopathologic, and molecular genetic steps. Pap smear examination, correlation with Human Papilloma Virus infection and many more biomarkers have gained importance. The discovery of HPV as a cause of cervical cancer, credited Harald Zur Hausen with the Nobel Prize in 2008. Persistent infection with a high oncogenic risk HPV, e.g., HPV 16 or HPV18, immunosuppression, certain HLA subtypes, oral contraceptives, use of nicotine are major risk factors.[1] Squamous cell carcinoma is the most common histologic subtype of cervical cancer, and High grade Squamous Intraepithelial Lesion (HSIL) is its immediate precursor.[2]

Host immune response, especially cytotoxic CD8-mediated response, is being closely studied in the pathogenesis and evolution of cervical intraepithelial neoplasia (CIN) and cervical cancer.[3,4] Programmed death receptor ligand 1 (PD-L1/B7-H1) is a recently described B7 family member. However, in contrast to B7-1 and B7-2, it does not interact with either CD28 or CTLA-4. Until now, one specific receptor has been identified that can be ligated by PD-L1 which has been shown to negatively regulate T-cell receptor (TCR) signaling. On ligating its receptor, PD-L1 decreases TCR-mediated proliferation and cytokine production. Normal tissue shows minimal surface expression of PD-L1 protein, while murine and human cancers show remarkable expression and can be further up-regulated upon IFN-γ stimulation.[5,6]

Recently, it has been suggested that therapy directed against PD L1 can cause a strong anti-tumour response in a wide variety of metastatic cancers. Cancers that do not respond to anti-PD L1 therapy show a strong CD8 T-cell presence in the untreated tumour with concomitant high PD L1 expression by immunohistochemistry.[7] PD L1 positive mononuclear cells are mostly present in the same histologic area as the dysplastic/neoplastic squamous cells also making this protein. Concomitant increase in expression of CD8 and PD L1 in a variety of stage IV cancers, including lung, colorectal, and renal cell has been demonstrated in different parts of world along with its association with poor prognosis of cancers. However, expression of the PD-1 ligands in solid tumours is still poorly understood.

METHODS

After obtaining approval from institutional ethics committee, we carried out a descriptive prospective study over one year, on 100 clinically diagnosed and histologically confirmed cases of premalignant and malignant lesions of the uterine cervix. 1/100 tissue was inadequate to read, hence statistical analyses has been done taking n=99.

Immunohistochemical expression of PDL1 antibody (DakoM 3653) in various stages of dysplastic lesions of cervix (CIN I/CINII/ CINIII) and squamous cell carcinoma (well, moderately, poorly differentiated) as diagnosed on histopathology were studied. Both percentage of tumour cells stained, and intensity of staining were noted. Staining patterns were compared amongst various pre-malignant and malignant histological types.

Hence the objective was to aid in research leading to development of monoclonal antibodies targeting programmed cell death 1 (PD-1) or its ligand (PD-L1) in cases of neoplasia of uterine cervix.

Patients not willing to give consent to be the part of the study or those under treatment of radiotherapy or chemotherapeutic agents were excluded.

Steps

- Detailed clinical evaluation with history and examination
- Appropriate radiological details to look for coexisting diseases or metastasis.
- Formalin fixed paraffin embedded tissue sections were stained with routine Haematoxylin and Eosin stain for histological diagnosis.
- Additional sections on coated slides were taken for Immunohistochemistry using PDL1 antibody which was performed according to manufacturer's instructions.

Immunohistochemistry Technique

1. Coated slides-3 aminopropyl triethoxy silane were used.
2. Fixed in hot plate (2 hrs).
3. Dewaxed in xylene (15 mins).
4. Hydration in graded alcohol (100%-->90%-->70%-->50%-->30%-->DW).
5. Antigen retrieval done by-
   Microwave-980C for 15 minutes in Tris EDTA buffer.
6. Cooled to room temperature (slowly)
7. Washed twice in Tris buffer saline
8. DAKO peroxidase blocking reagent (5 minutes)
9. Washed twice in TBS (5 minutes)
10. Primary antibody (90 minutes incubation)
11. Washed twice in TBS (5 minutes)
12. Secondary antibody (30 minutes incubation)
13. Washed twice in TBS (5 minutes)
14. Added DAB (3, 3'-diaminobenzidine tetrahydrochloride)
15. Washed twice in TBS (5 minutes)
16. Progressive staining with Haematoxylin
17. Washed and dehydrated by graded alcohol (70%-->90%-->100%).
18. Cleared with xylene and mounted with cover slips.

PD-L1 Immunostained Slides were Scored as

1. Intensity of staining
   0: negative
   1: weak
   2: moderate
   3: strong

2. % of tumour cells positive
   0: no tumour cell stained
   1+: <50%
   2+: 50-75%
   3+: >75

Grades

Low: Total score≤ 2;
Intermediate: Total score 3
Strong: Total score 4-6

Statistical Analysis

Statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 21.0 statistical Analysis Software. The values were represented in Number (%) and Mean ± SD.

RESULTS

36 cases were diagnosed as premalignant (13 CIN I, 9 CIN II, 14 CIN III); 63 as Squamous Cell Carcinoma (SCC); and 1 was inadequate for diagnosis

Tumour Type	Total (n=99)	Weak (Score ≤2) (n=15)		Intermediate (Score 3) (n=20)		Strong (Score 4-6) (n=64)	
		No.	%	No.	%	No.	%
Premalignant	36	10	66.7	6	30.0	20	31.2
Malignant	63	5	33.3	14	70.0	44	68.8

Table 1. Comparison of Grade of Premalignant and Malignant Tumours

χ^2=7.026 (df=2); p=0.020 (Sig)

CIN	Total (n=36)	Weak (Score ≤2) (n=10)		Intermediate (Score 3) (n=6)		Strong (Score 4-6) (n=20)	
		No.	%	No.	%	No.	%
CIN I	13	3	30.0	3	50.0	7	35.0
CIN II	9	2	20.0	2	33.3	5	25.0
CIN III	14	5	50.0	1	16.7	8	40.0

Table 2. Comparison among Premalignant Cases

χ^2=1.783 (df=4); p=0.776

Subtypes of Sq. Cell Carcinoma	Total (n=63)	Weak (Score ≤2) (n=5)		Intermediate (Score 3) (n=14)		Strong (Score 4-6) (n=44)	
		No.	%	No.	%	No.	%
Well diff.	12	1	20.0	5	35.7	6	13.6
Mod. diff.	48	4	80.0	9	64.3	35	79.5
Poorly diff.	3	0	0.0	0	0.0	3	6.8

Table 3. Comparison among Malignant Cases

χ^2=4.351(df=4); p=0.361

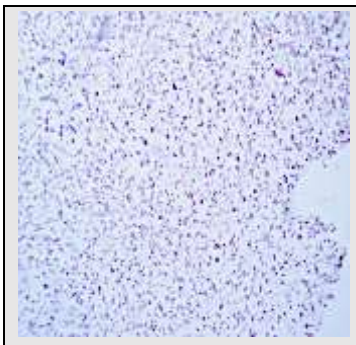

Figure 1. Low Power, PDL1 Positive Tumour Cells (> 90%)

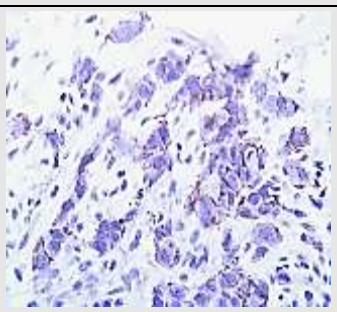

Figure 2. High Power View, PDL1 Membranous Positivity in Tumour Cells

DISCUSSION

Blank C et al in 2005 threw light on role of PD-L1 in tumour immune evasion. This review discussed in detail how T cells were regulated negatively through PD-1 in cases of tumours. They used the various data related to PD-1 work mechanism, published until then. Their work also focuses on blockade of various receptor sub-types, and whether this could be utilized in treating the tumours studied. The currently available data concerning negative T-cell regulation via PD-1, the blockade of PD-L1/PD-1 interactions, and the implications for adoptive T-cell therapies.[8]

Yang W et al in 2013 examined expression of PD-1 and PD-L1 on cervical T cells and dendritic cells (DCs), with CIN grades 0, I and II–III, on a set of 40 cases. Their work re-inforced the strong association of HPV 16 and cancer cervix, followed by the other virus types like 18, 53, 31 etc. PD-1 was frequently expressed on T cells while PD-L1 on dendritic cells. This expression increased in intensity and number of affected cells as dysplasia and cancer increased in grade. Thus their findings pave role in developing PD1/P-L1 pathway based immune treatment options in CIN and cancer associated with papilloma virus infection. The most common HPV type was HPV 16, followed by HPV 18, 33, 51 and 58. PD-1 and PD-L1 expression on cervical T cells and Dendritic Cells (DC), respectively, was associated with HR-HPV positivity and increased in parallel with increasing CIN grade. Thus up-regulation of inhibitory PD-1/PD-L1 pathway may negatively regulate cervical cell-mediated immunity to HPV and contribute to the progression of HR-HPV-related CIN and may aid in the development of PD-1/PD-L1 pathway-based strategies for immunotherapy of HR-HPV-related CIN.[9]

Louisa Mezache et al in 2015 observed negative PD L1 in normal cervical epithelia (0/55) even when adjacent to CIN or cancer, and increased PD L1 expression in CINs (20/21=95%) and SCC (56/70=80%); and localized to the dysplastic/neoplastic squamous cells and mononuclear cells, respectively. Significant increase in PD L1 detection in mononuclear cells was found when compared with ovarian and endometrial cancers. CD8 + lymphocyte was strongly concentrated around the dysplastic CIN cells and nests of invasive squamous cancer cells, further strengthening the hypothesis that anti-PD L1 therapy may have a role in the treatment of cervical cancer.[1]

Wu P et al published a meta-analysis in 2015 on a total of 3107 patients with solid tumour from 28 published studies. They correlated survival statistics in such patients with expression of PD-L1, computing Odds ratio in each participating study. Derived data was pooled employing random-effect model. Further statistical tools like publication bias and heterogeneity were attempted. Expression of PD-L1 and overall survival (OS) of patients with solid tumours, Odds ratios (ORs) from individual studies were calculated and pooled by using a random-effect model, and heterogeneity and publication bias analyses were also performed.

The researchers found 52.5% over-expression of PD-L1 as a median percentage. This correlated with poor outcome in terms of survival accounted at 3 (OR 2.43 with CI 1060-3.70) and 5 (OR 2.23 with CI 1.40) years post-diagnosis. p value was significant to three decimal places for both. When they analysed survival statistics of different solid tumours and their association with PD-L1, cancer oesophagus fared the worst while urothelial and colon cancer were at the other end of spectra. This study is very significant to literature as it helps us compare different parameters using PD-L1 expression in different tumour types.

The median percentage of solid tumours with PD-L1 overexpression was 52.5%. PD-L1 overexpression was associated with worse OS at both 3 years (OR = 2.43, 95% confidence interval (CI) = 1.60 to 3.70, P <0.0001) and 5 years (OR = 2.23, 95% CI = 1.40 to 3.55, P = 0.0008) of solid tumours. Among the tumour types, PD-L1 was associated with worse 3 year-OS of oesophageal cancer, gastric cancer, hepatocellular carcinoma, and urothelial cancer, and 5 year-OS of oesophageal cancer, gastric cancer and colorectal cancer.

These results suggest that expression of PD-L1 is associated with worse survival in solid tumours. However, the correlations between PD-L1 and prognosis are variant among different tumour types.[10]

Meng Y et al in 2018 performed a study to see the expression of PD-L1, PD-1, CD8 and HPV in cervical cancer and normal cervix by immunohistochemical staining. Their results showed that normal cervical tissue was less frequently positive for the immunomarkers used, when compared to cancer tissue. As the stage of cancer advanced with signs of metastatic potential, where histology revealed angioinvasion or draining lymph node positivity, frequency and intensity of immunostaining increased proportionately. In cases of prior adjuvant therapy received tissue, PD-L1 was over-expressed in tumour cells. Surrounding mononuclear cells over-expressed CD8 and PD1. This suggested immune checkpoint by PD-L1, role of Tumour Infiltrating Lymphocytes and immunotherapy mediated through PD-1/PD-L1 was more frequently positive for PD-L1, PD-1 and CD8 in cervical cancer tissues compared to normal tissues, especially those strongly stained HPV. Additionally, PD-L1, PD-1 and CD8 were more frequently stained in tissues from advanced tumour and tumour with lymphoid nodes or vascular invasion respectively. They found that tissues from patients with chemotherapy history had over expression of PD-L1 in tumour cells and more PD-1 and CD8 in stromal mononuclear cells, which were identified as tumour infiltrated lymphocytes (TILs). Their findings point to a key role of PD-L1 in immune escape of cervical cancer, and provide a rationale for therapeutic targeting of the PD-1/PD-L1 pathway.[11]

Carvalho MO et al in 2016 conducted a study taking 61 cases in tissue micro-array through immunohistochemistry for PD-L1, PD-L2 and CD8 using CD1a (antigen presenting cells) as an internal control. Their observation was interesting to the fact that statistically significant increase (almost thrice) was noted in CD8 expression in tumour cases, along with both PD-L1 and PD-L2. P values were significant in each comparison. However, cancer cases showed skewed CD1a expression in view of controls. Their findings pointed at loss of difference in immunoexpression in terms of stage of cervical cancer. In samples of invasive cervical cancers, there was a three-fold increase in the number of CD8 cells with an increase in the expression of both PD-L1 and PD-L2 (P<0.001 for each vs. control). A slight decrease in the number of CD1a cells was observed in malignant tissues compared to controls. This suggests that the immune response may be equivalent in early and late stage cervical cancers.[12]

Saglam O et al in 2018 worked on role of FDA approved drugs like pembrolizumab and nivolumab which work via pathways involving blockage of PD1/PDL1 receptors. These drugs were certified to be used in advance stage cancers. Cancer cervix, where squamous cell is the most commonly reported histological type, followed by adenocarcinoma express PDL1 in a significant percentage. Besides receptors, HPV status and Tumour Infiltrating Lymphocytes were other correlated variables in this study. The results of cervical cancer were compared with other gynaecological cancers viz endometrial and ovarian. PD1/PDL1 expression emerged as having strong role in cancer cervix. PD-1/PD-L1 blockage has become an important treatment modality after approval of pembrolizumab and nivolumab by Food and Drug Administration in advanced cancers. Recent studies provided support for usage of immune checkpoint inhibitors in advanced cervical cancer. Around 35% of cervical squamous cell carcinoma (C-SCC) and 17% of adenocarcinomas expressed PD-L1. Human Papilloma Virus status was also correlated with PD-L1 expression. PD-1/PD-L1 expression in tumour infiltrating inflammatory cells was higher in cervical

cancer in comparison to endometrial and ovarian adenocarcinomas.[13]

CONCLUSIONS

- Majority of premalignant cases fell in age group of <40 years and malignant cases >50 years.
- Common presenting complaints were bleeding per vagina (30.0%) followed by pain abdomen (14.0%), post-menopausal bleeding (12.0%), irregular menses (2.0%) and other complaints (4.0%). Out of 100 patients enrolled in our study 38.0% cases were asymptomatic.
- There was stronger expression of PD-L1 (membranous and cytoplasmic) in malignant cases as compared to premalignant cases.
- Range of score was 0 to 6. Median score of premalignant cases was 4.0 while that of malignant cases was 5.0. Mean score of Malignant cases (4.13±1.57) was found to be higher as compared to premalignant (3.14±2.14) cases.
- Difference in score and grade among premalignant and malignant cases was found to be statistically significant.
- Expression of PD-L1 can serve as a potent mechanism for potentially immunogenic tumours to escape from host immune responses.

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