**Measuring the impact of pathologic features of penile squamous cell carcinomas in PD-L1 expression: A machine learning approach**

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# INTRODUCTION

Penile squamous cell carcinoma (SCC) is a rare tumor for which few effective treatment options are available for advanced disease. Considering the significant morbidity of the standard treatment, identifying novel molecular and immunotherapeutic targets is actively sought. Programmed death-ligand 1 (PD-L1) is a coinhibitory molecule that impairs the T-cell response by down-regulating T-cell proliferation and cytokine production. Tumor cells often up-regulate PD-L1 and thereby evade the host immune system. Recently, immune-checkpoint inhibitors have been proven effective against several tumor types, but data on PD-L1 expression in penile SCC is scant with only few reports from low-incidence areas (1–5). In this study, we evaluate PD-L1 expression in a large dataset of patients with penile SCC from a high-incidence area.

# MATERIAL AND METHOD

The current study was approved by the Institutional Review Board at the Johns Hopkins School of Medicine (Baltimore, MD).

## Case selection and tissue microarray construction

The present study includes tissue samples from 108 patients with invasive squamous cell carcinoma of the. Cases were selected based on availability of formalin-fixed, paraffin-embedded tissue blocks. From each case, 1–4 blocks were selected. Four tissue microarrays (TMA) were built at the Johns Hopkins TMA Lab Core (Baltimore, MD) using a previously described procedure (6). Three tissue cores of 1 mm each were obtained per block, giving a representation of 3–12 spots per case. Normal tissue from various anatomical sites were included as control tissue. A total of 528 TMA spots were evaluated from the 108 cases.

## Morphologic evaluation

Pathologic features were evaluated using H&E-stained tissue sections. The following pathologic features were evaluated:

1. **Histologic subtype:** Histologic subtyping was carried out in whole tissue sections using the latest WHO criteria for classification of tumors of the urinary system and male genital organs (7).
2. **Histologic grade:** Histologic grading was carried out spot by spot using previously published and validated criteria (8). Briefly, grade 1 tumors were composed of well differentiated cells, almost undistinguishable from normal squamous cells except for the present of minimal basal/parabasal cell atypia. Grade 3 tumors were composed of any proportion of anaplastic cells showing nuclear pleomorphism, coarse chromatin, prominent nucleolus, irregular and thickened nuclear membrane, abundant and atypical mitoses. Grade 2 tumors corresponded to those cases not fitting criteria for grade 1 or grade 3 (i.e., it was an exclusion category).
3. **Host response:** Host response was evaluated spot by spot. Depending on the intensity of the inflammatory infiltrate observed, each spot was classified as showing no inflammation, mild inflammation, moderate inflammation, or intense inflammation.

## Immunohistochemistry

Immunohistochemical expression of PD-L1 was evaluated using a rabbit monoclonal anti–PD-L1 antibody (Cell Signaling, Boston). PD-L1 expression was evaluated in 2 cellular compartments, tumor cells and intratumoral lymphocytes. For tumor cells, we estimated percentages of positive tumor cells as well as their H-score, as previously described (1). For intratumoral lymphocytes, we estimated the percentage of positive tumor cells. Immunohistochemical evaluation was done spot by spot.

## Data analysis

Data was analyzed using Python 3.8 (Anaconda Distribution 2020.07, Anaconda, Inc., Austin, TX). Contingency tables were evaluated using the Pearson’s chi-square test. Correlation between numeric variables were evaluated using the Spearman’s rho coefficient. Numeric values were compared in groups using the Kruskal-Wallis test. A 2-tailed P < 0.01 was required for statistical significance. These statistical tests were implemented using the SciPy library (9).

To determine the impact that the selected pathologic features had on marker expression, linear regression models were built using machine learning. This impact was evaluated using the explained variance regression score. Machine learning models were built using the Scikit-learn library (10).

# RESULTS

## Pathologic features

The most common subtype, as expected was usual squamous cell carcinoma (45 cases), followed by warty-basaloid (24 cases), warty (16 cases) and basaloid (11 cases) carcinomas. Other subtypes included papillary (9 cases), verrucous (2 cases) and sarcomatoid (1 case) carcinomas. Grade 1 was observed in 51 spots, grade 2 was observed in 191 spots, and grade 3 was observed in 262 spots. This over-representation of grade 3 tumors is expected considering the geographical location of the patients. Tumors in patients from geographic areas of high incidence of penile cancer tend to be larger and of higher grade. In most cases, a host response was observed. In only 4 spots, no inflammatory cells were seen. In the remaining cases, mild inflammation was seen in 96 spots, moderate inflammation in 154 spots, and intense inflammation in 250 spots.

Basaloid and sarcomatoid carcinomas were entirely composed of grade 3 areas. Warty-basaloid and warty carcinomas were composed of predominantly grade 2 and grade 3 areas, while papillary and verrucous carcinoma were composed predominantly of grade 1 and grade 2 areas. Usual squamous cell carcinoma showed the heterogeneous aspect that it most common, with a mixture of histologic grades, predominantly grade 2 areas. This distribution pattern was totally consistent with the typical morphology of penile squamous cell carcinomas regarding histologic subtypes and grades. The association between histologic grade and histologic subtype was statistically significant (P<0.00001).

Intense inflammation predominated across histologic subtypes, with a similar pattern observed previously, with no significant differences between host response and histologic subtypes (P=0.24). Intense inflammation predominated in grade 2 and grade 3 tumors, followed by moderate inflammation and mild inflammation. In grade 1 tumors, proportions of mild, moderate and intense inflammation were similar. These differences were not statistically significant (P=0.22), indicating no association between histologic grade and host response.

## PD-L1 expression

### Overall expression

PD-L1 expression in tumor cells was evaluable in 504 spots. In tumor cells, mean expression was 26%, with a standard deviation of 34%. Median expression was 5%, with an interquartile range of 40%. The minimum value was 0% and the maximum value was 100%. PD-L1 expression in tumor cells showed a marked right-skewed distribution, suggesting that most values were very low. Considering >= 1% as the threshold for PD-L1 positivity, most spots (63%) were positive, compared to negative spots (37%). Two patterns of PD-L1 expression were observed in tumor cells. The predominant pattern was cytoplasmic and membranous (76%) with only cytoplasmic expression in the remaining cases (24%). Two patterns of PD-L1 expression were observed in tumor cells. The predominant pattern was cytoplasmic and membranous (250 spots) with only cytoplasmic expression in the remaining cases (81 spots).

Regarding H-scores, in tumor cells PD-L1 had a mean H-score of 36 with a standard deviation of 60. Median H-score was 5 and interquartile range was 50. The minimum and maximum values were 0 and 300, respectively. The distribution of H-scores showed the same right-skewed shape than with percentages, as expected.

PD-L1 expression in intratumoral lymphocytes was evaluable in 497 spots. In intratumoral lymphocytes, PD-L1 positivity was observed in a mean of 7 lymphocytes, with a standard deviation of 10 lymphocytes. The median number of positive PD-L1 intratumoral lymphocytes was 5, with an interquartile range of 9 lymphocytes. The minimum and maximum number of positive lymphocytes were 0 and 70, respectively.

A scatterplot of PD-L1 expression in tumor cells vs. intratumoral lymphocytes showed an apparent positive association. This positive association was confirmed using Spearman's correlation test, which showed a statistically significant, moderate positive correlation (rho=0.47, P<0.0001).

### Expression by pathologic features

**Histologic subtype:** When consider percentage of positive cells, higher expression of PD-L1 in tumor cells were noted for the sarcomatoid, basaloid and warty-basaloid subtypes (median of 100%, 25% and 20%, respectively). Low expression levels were noted in the usual and warty subtypes (5%), while the median expression was 0% for the papillary and verrucous subtypes. These differences were statistically significant (P<0.0001). The pattern of PD-L1 expression in tumor cells by histologic subtypes when using H-scores was similar to the expression pattern when using percentage of positive tumor cells. Similarly, these differences were statistically significant (P<0.0001). Regarding the association of histologic subtypes and PD-L1 expression in intratumoral lymphocytes, a pattern similar to PD-L1 expression in tumor cells was observed. These differences were also statistically significant (P<0.0001).

**Histologic grade:** Percentages of positive PD-L1 tumor cells increased from grade 1 to grade 2 to grade 3 tumors (median of 0%, 1% and 15%), suggesting an association between PD-L1 positivity in tumor cells and histologic grade. The Kruskal-Wallis test yielded a P<0.0001, indicating that the percentage differences were unlikely to be seen by chance alone. A similar pattern of PD-L1 expression measured by H-score and histologic grades was also observed, from a median of 0 points (grade 1) to 1 point (grade 2) to 18 points (grade 3). These differences were also statistically significant (P<0.0001). A similar trend was observed between histologic grade and PD-L1 expression in intratumoral lymphocytes (median percentages of 2%, 2% and 5%), but it did not reach the threshold for statistical significance (P=0.014).

**Host response:** Median percentage of PD-L1 positive tumors cells increased from mild to intense inflammation. Median PD-L1 positive tumors cells was 0% when no inflammation was seen, 1% with mild inflammation, 2% with moderate inflammation and 15% with intense inflammation. This association was statistically significant (P<0.0001). H-scores of PD-L1 expression tumor cells showed a similar pattern than with percentage of PD-L1 positive cells. The median H-score was 0 when no inflammation was seen, 1 in mild inflammation, 2 in moderate inflammation and 18 in intense inflammation. This association was statistically significant (P<0.0001). Median number of PD-L1 positive intratumoral lymphocytes increased from mild to intense inflammation. No PD-L1 positive intratumoral lymphocytes were identified when no inflammation was seen. Median number of PD-L1 positive intratumoral lymphocytes was 1% with mild inflammation, 2% with moderate inflammation and 5% with intense inflammation. This association was statistically significant (P<0.0001).

### Impact of pathologic features

For percentages of PD-L1 in tumor cells, histologic subtype explained 13.5% of PD-L1 variability, while histologic grade and host response explained 3.5% and 6.5% of the variability. When 2 features were combined, the highest explanatory power was for histologic subtype + host response (20%), followed by host response + histologic grade (9.6%) and histologic subtype + histologic grade (9.0%). When all 3 features were considered, they explained 16.3% of the PD-L1 variability in tumor cells. Overall, it seems that histologic subtype had the highest explanatory power for PD-L1 expression in tumor cells, while histologic grade had the lowest, either by themselves or in combination.

For H-scores of PD-L1 in tumor cells, histologic subtype explained 8.7% of all PD-L1 variability, while host response and histologic grade explained 3.4% and 1%. When 2 features were combined, the highest explanatory power was for histologic subtype + host response (13%), followed by histologic subtype + histologic grade (4.1%) and histologic grade + host response (4.1%). The explanatory power of all 3 features combined was 8.8%. Overall, the pattern observed with H-scores was like the pattern observed with percentage of positive cells.

In intratumoral lymphocytes, the scenario was different. First, when considered separately, the highest explanatory power was given by host response (11.2%), followed by histologic subtype (4.1%) and histologic grade (2.5%). When combining 2 features, the highest values were for host response + histologic grade (14.2%) and host response + histologic subtype (13.9%). Histologic subtype + histologic grade explained only 5.6% of the variability. Finally, when the 3 features were combined, the explanatory power was 16.3%, not a significant improvement. Thus, host response was the feature that explained better the variability in PD-L1 expression observed in intratumoral lymphocytes.

# DISCUSSION

In this study, we evaluated PD-L1 expression in a series of 108 penile squamous cell carcinomas using TMA. We evaluated PD-L1 expression in both tumor cells and intratumoral lymphocytes, taking into account pathologic features including histologic subtype, histologic grade and host response. We also built machine learning regression models to determine the explanatory power of these features in PD-L1 expression. PD-L1 expression (in both tumor cells and intratumoral lymphocytes) was associated with increasing histologic grades, with certain histologic subtypes such as sarcomatoid and basaloid carcinomas, and with increased inflammatory response by the host. In tumor cells, histologic subtype has the highest explanatory power for PD-L1 expression, followed by host response and histologic grade. In intratumoral lymphocytes, the landscape was different, with host response being the feature that better explained the variability in PD-L1 expression, followed by histologic subtype and histologic grade. However, the explanatory power of these features was low, indicating that other factors are in play and must be considered. Also, the practical implications that follow these findings include the emphasis that should be given to proper histologic subtyping and the necessity to include an evaluation of the inflammatory host response when diagnosing penile carcinomas.

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