Brief Report

Genetic Basis for PD-L1 Expression in Squamous Cell Carcinomas of the Cervix and Vulva

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IMPORTANCE Patients with squamous cell carcinoma (SCC) of the cervix or vulva have limited therapeutic options, and the potential for immunotherapy for this population has not been evaluated. Recent trials suggest that tumors with a genetic basis for PD-1 (programmed cell death protein 1) ligand expression are highly sensitive to therapeutic antibodies targeting PD-1.

OBJECTIVE To determine the genetic status of *CD274* (encoding PD-L1 [programmed cell death 1 ligand 1]) and *PDCD1LG2* (encoding PD-L2 [programmed cell death 1 ligand 2]) in SCCs of the cervix and vulva and to correlate the findings with PD-L1 protein expression.

DESIGN, SETTING, AND PARTICIPANTS We performed fluorescence in situ hybridization (FISH) using probes targeting *CD274*, *PDCD1LG2*, and the centromeric portion of chromosome 9, and immunohistochemistry (IHC) using an antibody recognizing PD-L1 on formalin-fixed, paraffin-embedded (FFPE) biopsy specimens from 48 cervical SCCs and 23 vulvar SCCs.

MAIN OUTCOMES AND MEASURES Tumors were categorized according to the genetic abnormality in *CD274* and *PDCD1LG2* (coamplification > cogain > polysomy > disomy) as detected by FISH, and evaluated on a semiquantitative scale (modified H score, the product of the percentage of tumor cells with positive staining and the maximum intensity of positive staining) for PD-L1 protein expression as detected by IHC.

RESULTS Overall, 71 samples of FFPE tissue from cases of cervical SCCs (n = 48) and vulvar SCCs (n = 23) were retrieved from the archives of Brigham and Women's Hospital and included in this study. We observed cogain or coamplification of *CD274* and *PDCD1LG2* in 32 of 48 cervical SCCs (67%) and 10 of 23 vulvar SCCs (43%). Median PD-L1 protein expression was highest among tumors with *CD274* and *PDCD1LG2* coamplification and lowest among tumors with disomy.

CONCLUSIONS AND RELEVANCE Recurrent copy number gain of the genes encoding the PD-1 ligands provides a genetic basis for PD-L1 expression in a subset of cervical and vulvar SCCs and identifies a class of patients that are rational candidates for therapies targeting PD-1.

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Tumors employ strategies of immune evasion to survive and spread.[1] A major mechanism involves expression of programmed cell death 1 ligands 1 and 2 (PD-L1, PD-L2) by tumor cells that bind PD-1 (programmed cell death protein 1) on effector T cells to suppress antitumor cellular immunity.[2] Clinical responses to PD-1 blockade are associated with PD-L1 expression by malignant tumor cells and provide a rationale for screening individual tumors to identify patients most likely to benefit.[3] However, the biological basis for the expression of the PD-1 ligands in solid tumors is poorly understood. We examined the integrity of the *CD274* (encoding PD-L1) and *PDCD1LG2* (encoding PD-L2) loci in a series of squamous cell carcinomas (SCC) of the cervix and vulva and correlated our findings with PD-L1 protein expression in the tumor.

Methods

Case Selection

Samples of formalin-fixed, paraffin-embedded (FFPE) tissue from cases of cervical SCCs (n=48) and vulvar SCCs (n=23) were retrieved from the archives of Brigham and Women's Hospital and included in this study. Additionally, data collected on cervical SCCs analyzed with a clinically deployed next-generation sequencing (NGS) assay were reviewed for evidence of selective copy gain of *CD274* and *PDCD1LG2* at 9p24 (eMethods in the Supplement).[4-6] Copy number analysis of *CD274* in cervical SCCs from The Cancer Genome Atlas (TCGA) (http://www.cbioportal.org; accessed 5/1/2015) (eTable 1 in the Supplement) was also obtained. Brigham and Women's Hospital provided institutional review board approval.

Fluorescence In Situ Hybridization

We performed fluorescence in situ hybridization (FISH) on FFPE sections of tumors with probes targeting *CD274*, *PDCD1LG2*, and the centromeric region of chromosome 9.[7]

Nuclei with a target:control ratio of greater or equal to 3:1 were scored as amplified; less than 3:1 but greater than 1:1 were scored as relative gain; and 1:1 as either polysomy (>2 copies), disomy (2 copies), or monosomy (<2 copies) (eMethods in the Supplement).[7] Cases were categorized according to the maximum genetic abnormality observed by FISH analysis (amplification > gain > polysomy > disomy).[7]

Immunohistochemistry

Immunohistochemistry was performed on all cases with a monoclonal antibody recognizing PD-L1[7-9] (eMethods in the

Figure 1. Copy Number Gain of *CD274* and *PDCD1LG2* in a Case of Cervical Squamous Cell Carcinoma

Copy number variation of targeted genetic loci on chromosome 9 as detected by a clinically deployed targeted sequencing assay (OncoPanel; Brigham and Women's Hospital). Each dot represents 1 targeted DNA segment (generally corresponding to 1 exon) arranged sequentially along the chromosome 9 from the p to q arm. C indicates centromere. The vertical axis is the ratio of the number of sequence reads for the specimen vs a panel of normals in log base 2 scale. A value of 0 denotes no difference from normal (diploid), and a log2 ratio greater than 1.5 indicates more than a 3-copy gain. Relative numbers of reads within *CD274* (cyan dots) and *PDCD1LG2* (orange dots) are indicated. One additional gene (*JAK2* [gray dots telomeric to *CD274*]) also showed copy gain by this assay, but additional genes on chromosome 9 did not.
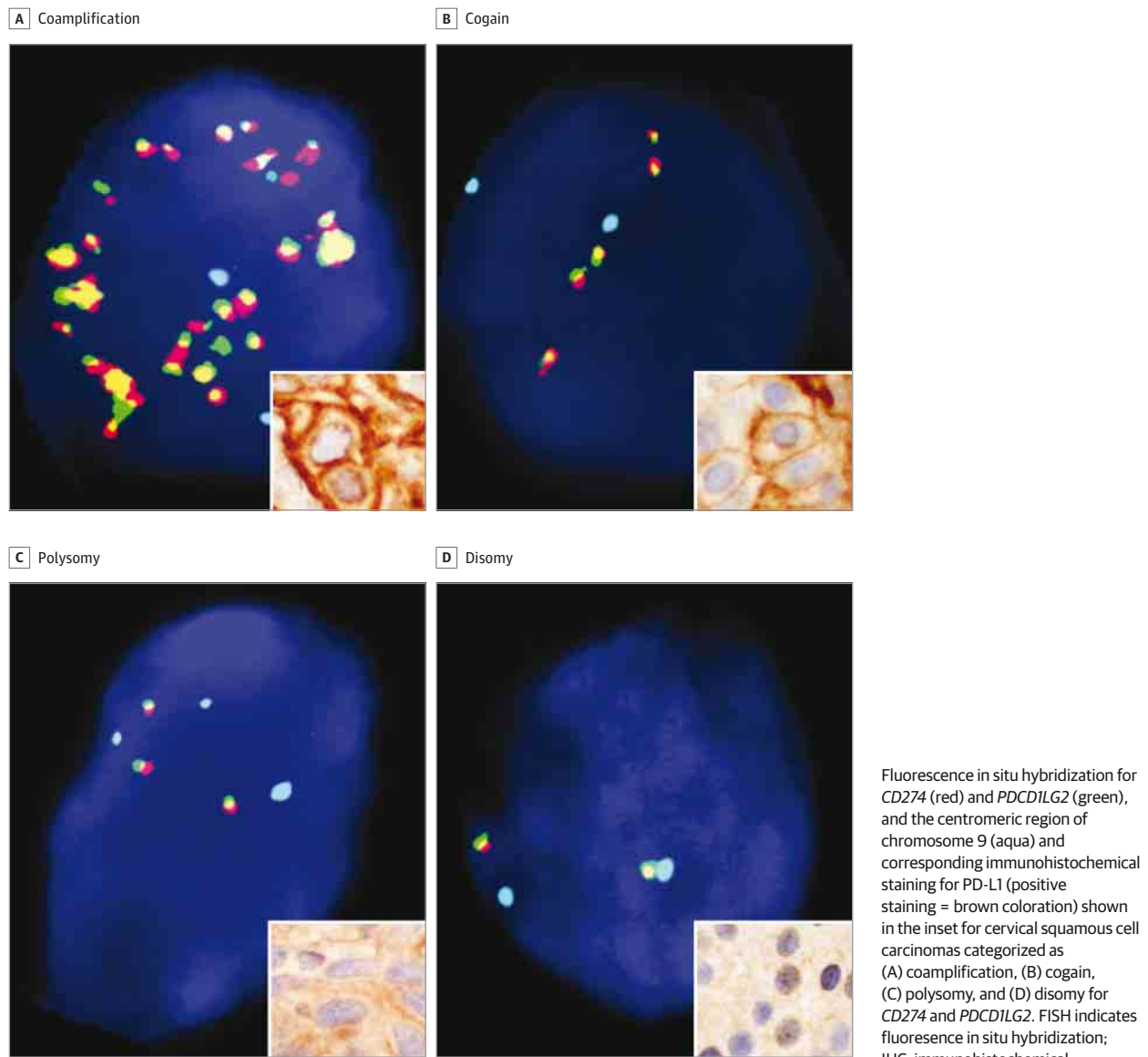
Figure 2. *CD274* and *PDCD1LG2* Status and PD-L1 Protein Expression in Cervical Squamous Cell Carcinoma With Copy Gain Identified by a Next-Generation Sequencing Assay

A | Fluorescence in situ hybridization

B | Immunohistochemical staining
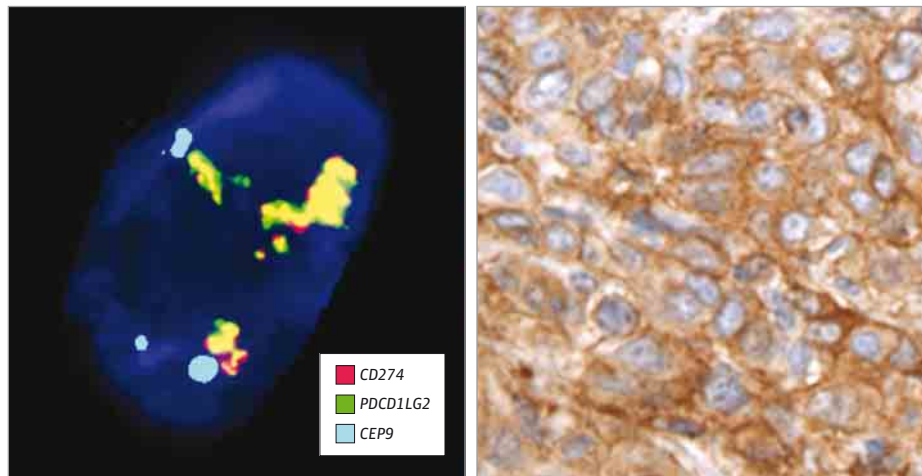
Formalin fixed paraffin embedded tissue from the cervical squamous cell carcinoma shown in Figure 1 (A) hybridized with probes targeting *CD274* (red), *PDCD1LG2* (green), and the centromeric region of chromosome 9 (aqua) and showing increased copies of *CD274* and *PDCD1LG2* relative to the centromeric region of chromosome 9, and (B) immunostained with anti-PD-L1 antibody and showing intense positive staining (brown coloration) of tumor cell membranes.

Figure 3. Examples of *CD274* and *PDCD1LG2* FISH and PD-L1 IHC in Cervical Squamous Cell Carcinomas

Fluorescence in situ hybridization for *CD274* (red) and *PDCD1LG2* (green), and the centromeric region of chromosome 9 (aqua) and corresponding immunohistochemical staining for PD-L1 (positive staining = brown coloration) shown in the inset for cervical squamous cell carcinomas categorized as (A) coamplification, (B) cogain, (C) polysomy, and (D) disomy for *CD274* and *PDCD1LG2*. FISH indicates fluoresence in situ hybridization; IHC, immunohistochemical.

Supplement). PD-L1 stained slides were scored by both maximal intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong) and percentage of tumors cells positive (0%-100%; with any intensity of positive staining) to generate a modified H score (range, 0-300).

Statistics

Data were summarized descriptively. The exact Kruskal-Wallis test was applied across the ordered categories of dysregulation (disomy, polysomy, cogain, and coamplification) to assess changes in H score related to degree of dysregulation. Graphs were generated with GraphPad Prism 5 software (GraphPad Software, Inc), and *P* values less than .05 were considered statistically significant; there was no adjustment for multiplicity of testing.

Results

Cervical Squamous Cell Carcinoma

The NGS assay revealed 3 of 24 (12.5%) cervical SCCs with copy gains of *CD274* and *PDCD1LG2* at chromosome 9p24.1 (**Figure 1** and data not shown). One case had tissue available for additional analyses, and we performed FISH using probes targeting *CD274* and *PDCD1LG2*.[7] We observed high level coamplification of *CD274* and *PDCD1LG2* in 96% of the tumor cells, with up to 15 copies of both genes per cell (**Figure 2**A). Cogain of *CD274* and *PDCD1LG2* was present in the remaining 4% of tumor cells analyzed. Immunohistochemical staining with an antibody specific for PD-L1 revealed robust protein expression (2+ intensity) in 95% of

the tumor cells (modified H score, 190)[8] with predominantly membranous staining pattern (Figure 2B).

We then evaluated an additional cohort of 47 cervical SCCs lacking previous genomic annotation (for a total of 48 cervical SCCs) with the FISH assay. We observed coamplification or cogain of *CD274* and *PDCD1LG2* in 32 of 48 (67%) cases (**Figures 3** and **4**). Overall, 9 of 48 (19%) cases were categorized as polysomic and 7 of 48 (15%) disomic for chromosome 9. There was copy number heterogeneity within each category. Cases with tumor cells showing coamplification of *CD274* and *PDCD1LG2*, and categorized as such, also included malignant cells with lesser degrees of gain for *CD274* and *PDCD1LG2* (cogain, polysomy, and disomy). Similarly, tumors categorized as cogain included malignant cells with polysomy and disomy. Occasional cases included malignant cells with relative loss of *CD274* and *PDCD1LG2* or monosomy 9 (eTable 2 in the Supplement). In no case did we observe discordant alterations of *CD274* and *PDCD1LG2*.

Independent scoring of immunohistochemistry for all cases revealed a range of PD-L1 protein expression across tumors with highest median expression in cases with coamplification of *CD274* and *PDCD1LG2* and lowest in cases with disomy (Figures 3 and 4) (eFigure 1 in the Supplement). PD-L1 expression, when observed, was accentuated at the cell membrane of the tumor cells (Figure 3).

For independent validation of the genetic alterations we observed in our cohort of cases, we interrogated TCGA data for cervical SCCs and found evidence for amplification or gain of *CD274* in 28 of 129 (22%) cases (eTable 1 in Supplement).

Vulvar Squamous Cell Carcinoma

While cervical SCCs are associated with high-risk human papilloma virus (HPV) in the great majority of cases,[10] vulvar SCCs have both HPV-related and HPV-unrelated mechanisms of pathogenesis. We examined a series of 23 vulvar SCCs using p16(INK4a) as a sensitive and specific surrogate biomarker of high-risk HPV infection.[11,12] Overall, 6 cases (26%) showed coamplification of *CD274* and *PDCD1GL2*, 4 cases (17%) showed cogain, 6 cases (26%) showed polysomy, and 7 cases (30%) showed disomy (**Figure 5**A). Vulvar SCCs positive for p16 (n = 7

[30%]) included 2 cases with cogain of *CD274* and *PDCD1LG2*, 1 with polysomy, and 4 with disomy (Figure 5A). Vulvar SCCs negative for p16 (n = 16 [70%]) included 6 cases with coamplification, 2 with cogain, 5 with polysomy, and 3 with disomy. There was no association between p16 status and the genetic category of the tumor (P = .08). Immunohistochemical staining for PD-L1 across all cases revealed the highest median PD-L1 protein expression among cases with coamplification of *CD274* and *PDCD1LG2* and decreasing values with decreasing genetic complexity (Figure 5B) (eFigure 2 in the Supplement).

Discussion

Our data reveal that selective copy number gain of *CD274* and *PDCD1LG2* at 9p24.1 occurs frequently in SCCs of the cervix and vulva and provides a genetic basis for increased PD-L1

Figure 4. Levels of PD-L1 Expression by Genetic Category in Cervical Squamous Cell Carcinoma
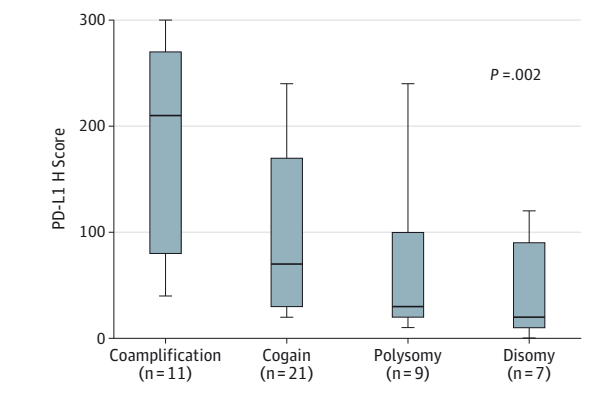
PD-L1 H scores by genetic category in cervical squamous cell carcinomas demonstrated by box and whisker plots showing median score (horizonal line), 25th to 75th percentiles (boxes), and minimum and maximum scores (whiskers). H scores are the product of the percentage of tumor cells with positive staining (0-100) and the maximum intensity of positive staining (1, 2, or 3).

Figure 5. p16[INK4A] Expression and Levels of PD-L1 Expression by Genetic Category in Vulvar Squamous Cell Carcinomas
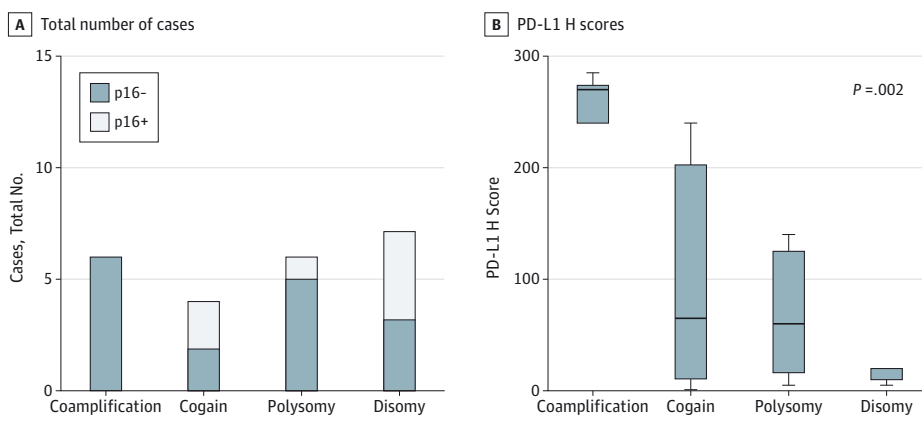
A, Total number of cases within each genetic category with numbers of p16− cases in darker shade. B, Box and whisker plots showing median (horizontal line), 25th to 75th percentiles (boxes), and minimum and maximum H scores (whiskers) for tumors analyzed within each genetic category (P = .002). H scores are the product of the percentage of tumor cells with positive staining (0-100) and the maximum intensity of positive staining (1, 2, or 3).

protein expression in these tumor types. To date, the most detailed analysis of 9p24.1 alterations has been in classical Hodgkin lymphoma (cHL), in which malignant Reed-Sternberg cells have 9p24.1/*CD274/PDCD1LG2* copy gain and increased expression of the PD-1 ligands.[6] We have also reported that Epstein-Barr virus–encoded signaling proteins contribute to PD-1 ligand expression in primary cells.[13,14] It remains to be established whether HPV-encoded proteins have a similar role in SCCs.[15] However, the data presented here suggest that 9p24.1 gene copy number alterations are a major mechanism of increased PD-L1 expression in SCCs of the cervix and vulva, irrespective of viral infection.

A recent trial[7] of single agent nivolumab in patients with relapsed and/or refractory cHL revealed the highest overall re-sponse rate (87%) reported to date for an individual tumor type. Tissue biopsies were available for a subset of patients in this trial, and all showed copy gain of *CD274* and *PDCD1LG2*. These results suggest that tumors with a genetic basis for PD-1 li-gand expression may be uniquely sensitive to PD-1 blockade.[7]

Conclusions

We show that cogain or coamplification of *CD274* and *PDCD1LG2* is common in cervical and vulvar SCCs, and pro-vides a genetic basis for PD-L1 expression. Thus, a significant proportion of patients with these tumors are rational candi-dates for clinical trials of PD-1 blockade.

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