Evidence for Efficacy of Treatment With the Anti-PD-1 Mab Nivolumab in Radiation and Multichemorefractory Advanced Penile Squamous Cell Carcinoma

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Summary: Penile squamous cell carcinoma (PeSCC) is a rare tumor and advanced PeSCC is associated with poor survival due to the aggressiveness of the disease and lack of effective systemic therapies. We describe for the first time a case with advanced chemoradiation refractory PeSCC who had documented response to active immunotherapy with the immune checkpoint inhibitor, anti-programmed death-1 monoclonal antibody Nivolumab. The patient suffered from a poor prognosis human papillomavirus-negative PeSCC, with a somatic inactivation mutation of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene in tumor cells, and treatment with Nivolumab resulted in a partial response to therapy and significant tumor shrinkage. Histology transitions and alterations in tumor-infiltrating cytotoxic $CD8^+$ T-cell lymphocytes, programmed death ligand-1 expression on tumor cells and immune cells in tumor lesion biopsies pretreatment and posttreatment with Nivolumab were observed and described. In conclusion, in patients with metastatic PeSCC active immunotherapy combinations with an anti-programmed death-1/programmed death ligand-1 agent may be beneficial and further relative clinical studies are required.

Key Words: penile squamous cell carcinoma, nivolumab, immune checkpoint inhibitors, PD-L1, PD-1, tumor-infiltrating lymphocytes

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CASE REPORT

A 47-year-old Caucasian man presented in July 2015 with relapsed poorly differentiated high-grade (grade 3) squamous penile carcinoma (penile squamous cell carcinoma—PeSCC) with metastasis at inguinal and iliac lymph nodes. The patient had no significant past medical history other than cancer and Eastern Cooperative Oncology Group (ECOG) performance status of 0–1. He did not smoke or consume alcohol and worked as a farmer. The patient was not circumcised and no pertinent history of human papillomavirus (HPV) infection was mentioned. There was no family history for neoplastic diseases. The patient had a long medical record regarding his present illness starting from August 2011 where a highly dysplastic inflammatory lesion on glans penis and prepuce was successfully resected. On February 2013 the patient presented with a local relapse of the tumor which was marginally excised. The lesion histology demonstrated inflammation with intraepithelial squamous neoplasia (grade 1–3) and focal infiltration by squamous carcinoma of high differentiation (grade 1) (T1a). Afterwards the patient received prophylactic radiotherapy (total dose: 64 Gy/32 Fr; whole pelvis 44 Gy/22 Fr, boost 20 Gy/10 Fr). In April 2015, a new locally extended tumor relapse was diagnosed (T1bN0). The patient underwent local surgery with bilateral resection of inguinal lymph nodes (superficial for N0 disease). The tumor histology diagnosed infiltrative squamous carcinoma of high and moderate differentiation (grade 1–2). The patient presented again in July 2015 with a new relapse and metastasis to bilateral inguinal and right iliac lymph nodes. The tumor was an advanced PeSCC as the tumor biopsy sections demonstrated lymph node infiltration by a high-grade (grade 3) squamous penile carcinoma. The patient opted out of salvage surgery. From July 2015 to February 2016, the patient received sequential chemotherapy with cisplatin and 5-fluorouracil, carboplatin and capecitabine, docetaxel and gemcitabine unsuccessfully with continuous progression of the disease. New tumor biopsy was performed and showed again infiltration by a high-grade (grade 3) squamous penile carcinoma (Fig. 1A). In February 2016, the patient received supplementary radiation therapy (5 sessions every other day, volumetric modulated arc radiotherapy—image-guided radiation therapy technique, in the disease site with 2 treatment arcs, daily dose: 7.0 Gy, total dose: 35 Gy) synchronously with chemotherapy (carboplatin/vinorelbine). In April 2016 further progression of disease was diagnosed and immunotherapy with the anti-programmed death-1 (PD-1) Mab Nivolumab was initiated at 3 mg/kg every 14 days and was administrated to the patient for 8 cycles. The patient had a very good tolerance and excellent tumor partial response to the Nivolumab therapy (Fig. 2). Partial response with decrease of the longer diameters of target tumor lesions from 10.1 to 2.8 cm and from 5.6 to 1.0 cm in right and left inguinal lymph node metastatic blocks was observed in magnetic resonance imaging scans, respectively (at least a 30% decrease in the sum of diameters of target lesions; RESIST criteria version 1.1), as well as, $>80\%$ reduction of tumor volume was obtained (Figs. 2B, D, F). New tumor biopsy and histologic examination following the treatment with Nivolumab, confirmed the clinical response and demonstrated tumor necrosis in pathology findings. In November 2016 the patient presented with an extensive postradiation dermatitis and cellulitis which was rapidly followed by regional skin ulceration, necrosis, and infection. In January 2017 the patient suffered from necrotizing fasciitis (Fournier gangrene) and died from septic shock in February 2017. Response to treatment with Nivolumab was observed 2.5 months after initiation of therapy and endured over 6 months until the patient died. Treatment with Nivolumab was well tolerated and produced no grade 2–4 or other unexpected treatment-related toxicity.

Molecular Testing and Screening

Biopsy sections were taken before chemotherapy treatment in July 2015 and before immunotherapy in February 2016 and were both molecularly and immunohistochemically analyzed. The analysis of the biopsy material for HPV infection regarding the amplification and detection of the HPV genomic L1 region by polymerase chain reaction procedures was negative. Extensive immunohistochemical analysis of possible predictive biomarkers in the cancer tissue sections revealed negative expression for *TEL3*, *p16*, *HER2*, positive expression for

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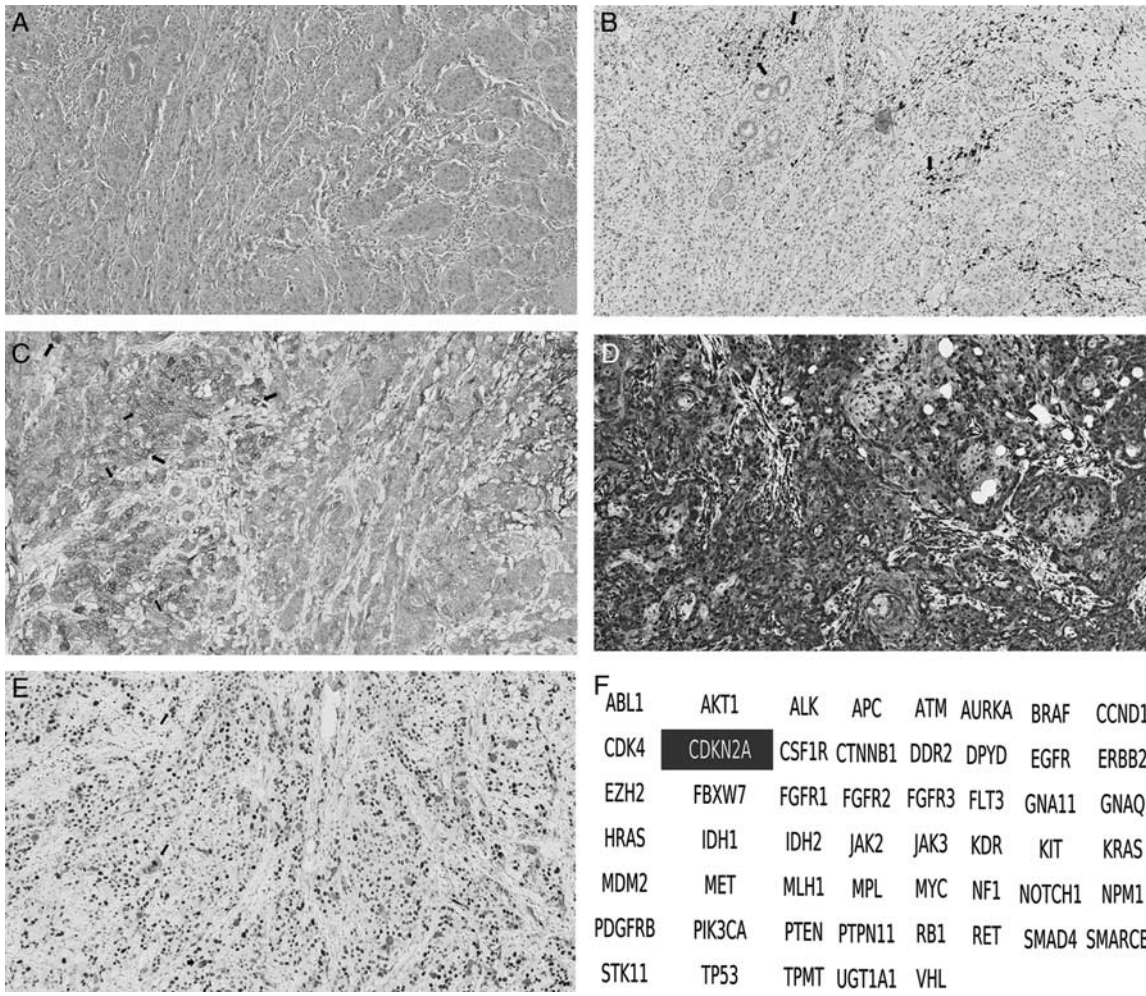

FIGURE 1. A, Histology of penile squamous cell carcinoma (SCC) with mostly poorly differentiated (grade 3) and less moderately differentiated (grade 2) cancer cells (hematoxylin and eosin stain). B, Positive expression of tumor infiltrating cytotoxic $CD8^+$ T lymphocytes (5%–10% of $CD8^+$ tumor-infiltrating lymphocytes/mm^2) (C8/144B clone; Dako). C, Positive expression of PD-L1 on $\geq 5\%$ of tumor cells (PD-L1 IHC 28-8 pharmDx; Dako). D, High expression of *pERK1/2* in tumor cells (clone 20G11, GSTY 4376 Rabbit Mab; Cell Signaling Technology). E, Positive expression of pRb in tumor cells [Rabbit polyclonal Phospho-*Rb* (Ser780) Ab (ab47763); Abcam], before treatment with Nivolumab (original magnification, ×50). F, Next-generation sequencing screening for genomic alterations in 65 genes of tumor tissue indicating mutation on *CDKN2A*.

PTEN, CD8 cytotoxic T cells—tumor-infiltrating lymphocytes (TILs)—(percentage marked cells ≈10%/mm^2) (Fig. 1B) and high expression of programmed death ligand-1 (PD-L1) (percentage marked cells score ≈10%) (Fig. 1C), thymidylate synthase, *TUBB3*, *ERCC1*, *RRMI*, *pERK1/2* (Fig. 1D), and pRb (Fig. 1D). Next-generation sequencing genomic study was performed for a panel of 65 possibly related genes in the cancer tissue and showed mutation of *CDKN2A* (cDNA: c.172C > T–AA: p.R58* VarFreq: 40%) (Fig. 1F). Immunochemistry was performed in multiple tumor biopsy sections. Activated CD8 T lymphocytes (TILs) were analyzed in 12–15 representative fields in each of multiple (6–8) pretreatment and posttreatment sections of tumor biopsies in order to obtain statistical significance ($P < 0.05$). Moreover, it was not observed microsatellite instability (MSI) or mutation/s related to sensitivity or resistance to immunotherapy. For MSI testing, MSI Analysis System version 1.2 from Promega was used according to the manufacturer's data sheet. The result for this biopsy was comparable to the negative control and therefore MSI was considered as negative or low. However, a high tumor mutation burden (TMB) was performed with next-generation sequencing study. The TMB is originally defined by exome sequencing as the number of mutation by mega base (Mb). After the analysis of several cancer types, it has been reported that the median TMB is 3.6 mutations/Mb with a range of 0–1241 mutations/Mb. This median is cancer and age dependent (the median of the TMB at 10 y is 1.67 mutations/Mb and 4.5 at 88 y). TMB was calculated using a nonexome sequencing panel as previously has been reported.[1] The tested tumor sample showed 138.7 variants/Mb (5 variants) while the median for this cancer type is 83.4 variants/Mb (3 variants). Notably, the vast majority of MSI-high tumors have also a high TMB (83%), and 97% with a TMB >9 mutations/Mb. However, the opposite is not true, only 16% of samples with high TMB were classified as MSI-high.

Histologic and immunohistochemical examination performed in tumor biopsy sections following the treatment with Nivolumab demonstrated extensive tumor necrosis and increased inflammatory reaction, tumor malignancy downgrading (grade 1–2) (Figs. 3A–D). A significant rise of tumor-infiltrating cytotoxic $CD8^+$ T lymphocytes ($\geq 20\%$) in tumor lesions after treatment with Nivolumab was showed (Figs. 3E, F). Moreover, a significant attenuation of the PD-L1 expression on tumor cells (percentage marked cells: $\leq 1\%$) and significant rise of the PD-L1 expression on the infiltrating inflammatory mononuclear cells was observed (Figs. 3G, H).

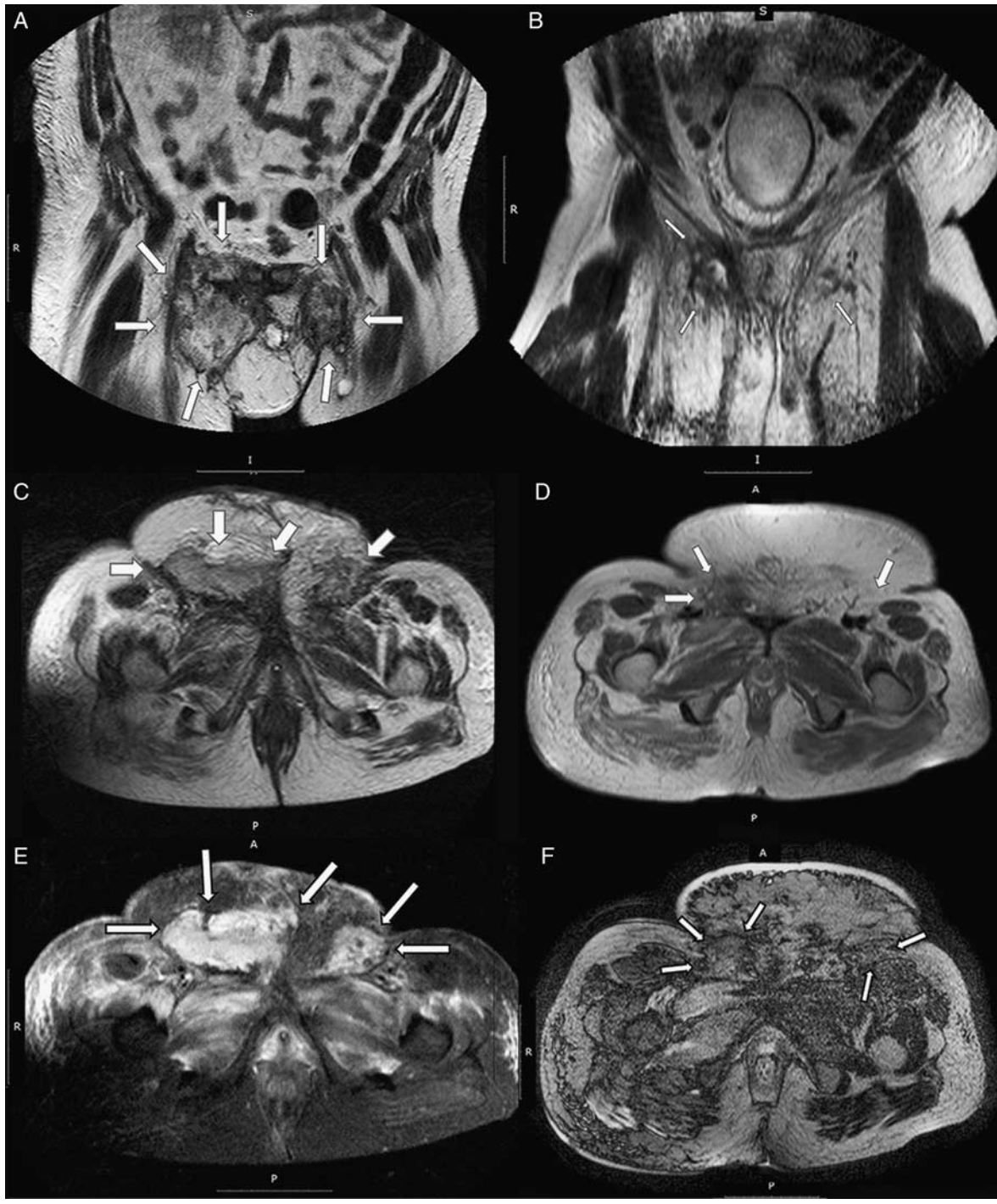

FIGURE 2. Magnetic resonance imaging (4/2016): T2w coronal (A), axial (C), and STIR axial (E) images; bilateral enlarged inguinal lymph nodes due to progression after cytotoxic chemotherapy and radiation therapy, before treatment with Nivolumab. Magnetic resonance imaging (9/2016): T2w coronal (B), axial (D), and STIR axial (F) images. High signal (possibly necrotic tissue) shrunk right inguinal lymph nodes due to response to immunotherapy with Nivolumab. Tumor lesions are indicated with white arrows.

DISCUSSION AND CONCLUSIONS

PeSCC incidence rates varying in the range of 1–10 cases per 100,000 men depending on ethnicity, geographic area, cultural background, and social habits and is considered as a rare disease. Advanced PeSCC (T4, N2–3, and M1) is associated with poor survival due to the aggressiveness of the disease and lack of effective systemic therapies. PeSCC is primarily treated by surgical resection while locally advanced and metastatic disease requires a multidisciplinary treatment approach. However, mortality and morbidity remain high, as standard treatment with cytotoxic chemotherapy regimens and/or radiation therapy

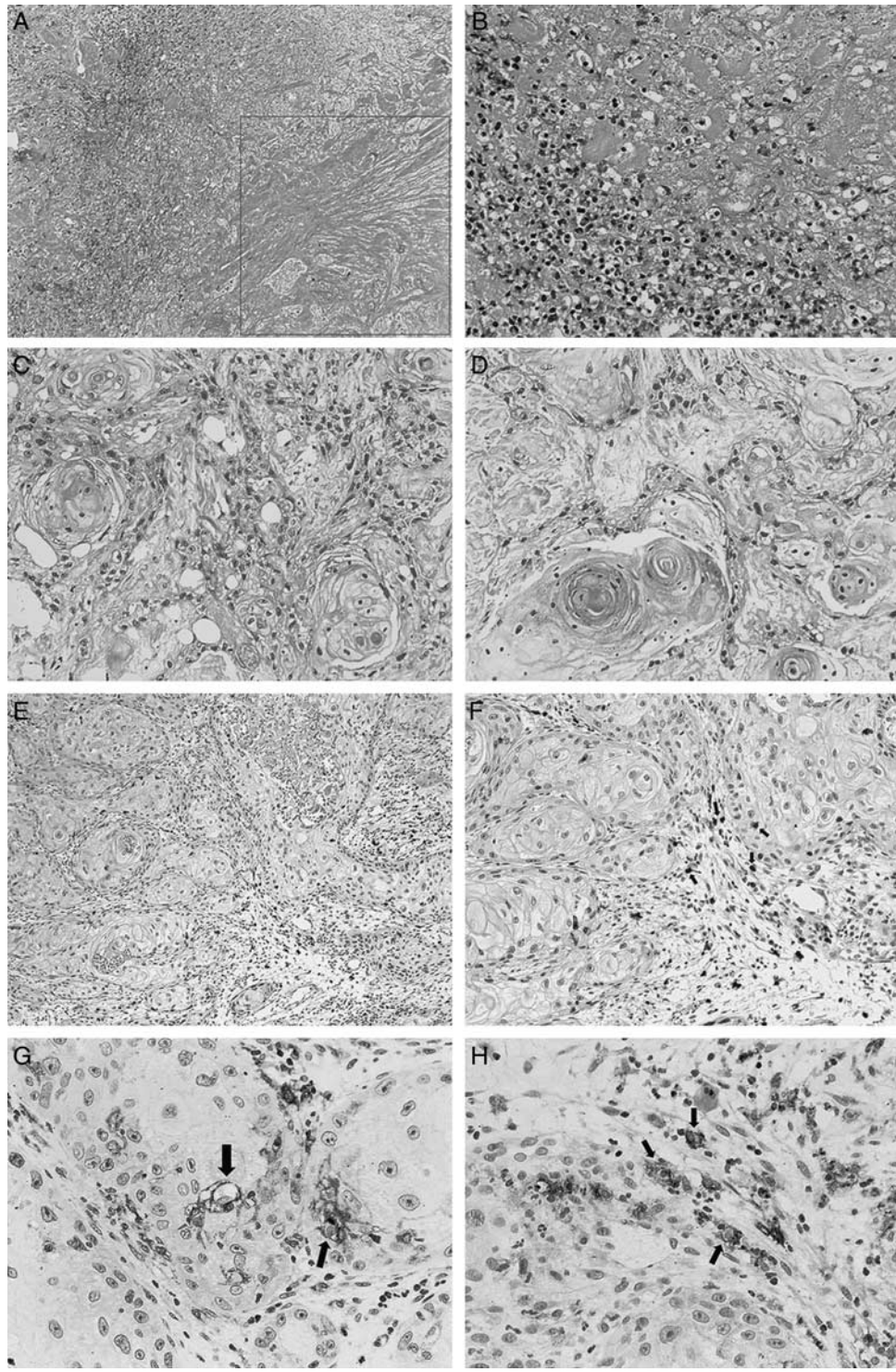

FIGURE 3. A, Microphotograph taken from biopsy obtained after treatment with Nivolumab showing extensive tumor necrosis (inset) with focal mixed inflammatory infiltration. Stain, hematoxylin and eosin; original magnification, ×50. B, Microphotograph focusing on inflammation of tumor necrosis presenting mixed inflammatory type of polymorphonuclear and mononuclear leukocytes and immune cells after treatment with nivolumab. Stain, hematoxylin and eosin; original magnification, ×200. C and D, Microphotographs of residual tumor cell nests after treatment with nivolumab presenting downgrading tumor histology with low to moderate grade (1–2) squamous cell carcinoma cells with degenerative necrosis and focal deposition of keratin. Stain, hematoxylin and eosin; original magnification, ×200. E and F, Microphotographs that demonstrate a rise of tumor infiltrating cytotoxic $CD8^+$ T lymphocytes ($\geq 20\%$) in tumor lesions after treatment with Nivolumab (C8/144B clone; Dako); original magnification, ×100, ×200. G and H, Microphotographs of residual tumor cell nests after treatment with nivolumab showing attenuation of PD-L1 expression on tumor cells and significant augmentation of PD-L1 expression on immune cellular elements surrounding tumor cells (PD-L1 IHC 28-8 pharmDx; Dako); original magnification, ×400. Cells expressing PD-L1 are showed with black arrows.

resulted poor clinical outcome, especially in patients with HPV-negative tumors.[2]

To our knowledge, we describe for first time a case with advanced chemoradiation refractory PeSCC who had documented response to active immunotherapy with the immune checkpoint inhibitor, anti-PD-1 monoclonal antibody Nivolumab. The patient suffered from a poor prognosis HPV-negative PeSCC, nesting a somatic inactivation mutation of cyclin-dependent kinase Inhibitor 2A (*CDKN2A*) gene in tumor cells, and treatment with Nivolumab resulted in a very good response to therapy and significant tumor shrinkage. Pretreatment tumor biopsy presented positive expression of tumor infiltrating cytotoxic $CD8^+$ T lymphocytes ($CD8^+$ TILs), positive expression of PD-L1 on $\geq 5\%$ of tumor cells, and high expression of phosphorylated extracellular signal–regulated kinases *pERK1/2* and retinoblastoma tumor suppressing protein pRb in tumor cells, indicating for mixed features on tumor aggressiveness and growth (Fig. 1). Moreover, tumor cells did not show expression of *p16* (in concordance to HPV negativity). MSI absence and high TMB were additional molecular characteristics of the tumor. Treatment with Nivolumab resulted in significant changes in tumor histology such as, inflammatory tumor necrosis, downgrading of tumor malignancy with degenerative necrosis and focal deposition of keratin (Figs. 3A–D). Moreover, after treatment with Nivolumab, a significant increase of TILs, a significant enhancement of PD-L1 positively expressing immune-cells (T cells, macrophages) in tumor lesions (Figs. 3E, F) and a significant attenuation of PD-L1 expression on tumor cells was demonstrated (Figs. 3G, H).

In advanced PeSCC the most common relevant genomic alterations identified were the *CDKN2A* point mutations and homozygous deletion (40%), *NOTCH1* point mutations and rearrangements (25%), *PIK3CA* point mutations and amplification (25%), *EGFR* amplification (20%), *CCND1* amplification (20%), *BRCA2* insertions/deletions (10%), *RICTOR* amplifications (10%), and *FBXW7* point mutations (10%).[3] Somatic mutations of *CDKN2A* are common in the majority of human cancers, with estimates suggesting that *CDKN2A* is the second most commonly inactivated gene in cancer after *p53*. This gene encodes for 2 proteins, the *INK4* family member *p16* (or *p16INK4a*) and the *p14arf*. Both act as tumor suppressors by regulating the cell cycle. *p16* inhibits *CDK4* and *CDK6* and thereby activates the retinoblastoma (*Rb*) family of proteins, which block transition from G1 to S-phase.

The tumor molecular profile of predictive biomarkers was compatible to its resistance to cytotoxic chemotherapy (negative expression for *TEL3* and high expression of thymidylate synthase, *TUBB3*, *ERCC1*, *RRMI*), while tumor cell PD-L1 positive expression, high TMB and high percentage of TILs CD8 cytotoxic T cells predicted response to active immunotherapy with anti-PD-1/PD-L1 monoclonal antibodies.[3] In our case, after treatment with Nivolumab tumor cells showed significant restriction of PD-L1 expression but notably, a significant accrual of PD-L1 expression in tumor infiltrating immune cells was observed. In some clinical studies, PD-L1 expression on infiltrating immune cells was more widespread and can be even a stronger predictor for treatment response than PD-L1 expressions on tumor cells alone.[4]

Pathogenesis of PeSCC is associate with multiple causative factors including tobacco toxins, UV radiation, household contaminants from solid fuel combustion, which have been also implicated in carcinoma of the cervix, as well as promoting factors, such as cytokines related to chronic inflammation and high-risk HPV, mainly HPV-16 and HPV-18, as well as other inflammatory conditions such as chronic balanitis, lichen sclerosus, and phimosis are also implicated in penile cancer etiology. In patients with PeSCC, keratinizing squamous cell and verrucous lesions harbor high-risk HPV in 30% of cases coexisting with squamous cell hyperplasia and/or lichen sclerosus, while basaloid and warty carcinomas may harbor HPV in 80%–100% of cases. HPV infection is associated with significantly better survival rate. HPV-positive tumors express more frequently HER3 and cytoplasmic Akt1, while HPV-negative tumors express more frequently phosphorylated *EGFR*, which is related to a negative prognostic effect. Thus, based on the established etiopathogenetic role of HPV in a subgroup of PeSCC patients, HPV-associated antigens may provide specific targets for an immunotherapy approach in penile cancer.[5]

The PD-1/PD-L1 axis has been demonstrated to play an important role in tumor immune escape, and immunotherapies targeting this pathway have shown great success in certain cancer types. The expression of immune-checkpoint biomarkers in PeSCC has been studied and demonstrated that the majority of primary PeSCCs (40%–62%) were positive for PD-L1 expression, with a strong positive correlation of PD-L1 expression in primary and metastatic samples. High PD-L1 expression in tumor cells was associated with a poor prognosis and high-risk clinicopathologic features, while PD-L1 expression in tumor cells was significantly associated with the extent $CD8^+$ TILs. It has been reported that only activated lymphocytes express PD-1 that might lead to the expression of PD-L1 by the tumor cells. Therefore, the presence of such population of lymphocytes ($CD8^+$) has been associated with better clinical outcomes for immunotherapy. Further, it was shown that during a therapy, an increase in $CD8^+$ cells in serial tumor samples was associated with better response. However, testing $CD8^+$ T-cell infiltration alone might not have a big predictive value because CD8 expression is affected by tumor heterogeneity and temporal variability. The upregulation of PD-L1 in PeSCC include both extrinsic and intrinsic mechanisms. These findings indicated that the PD-1/PD-L1 axis might be a potential therapeutic target for patients with PeSCC and provide a rational basis for further investigation of anti-PD-1 and anti-PD-L1 targeting immune-checkpoint inhibitors in patients with advanced PeSCC.[4–9]

Specific active immunotherapy checkpoint inhibitors include monoclonal antibodies which target immune checkpoint receptors on T cells or tumor cells such as PD-1, PD-L1, cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), lymphocyte function-associated antigen 3 (LFA-3). Since 2010, numerous trials on different tumor types have been conducted, which has resulted in the approval of these drugs for the treatment of melanoma, lung cancer, Hodgkin lymphoma and head and neck cancers. Urothelial and renal-cell carcinomas are immunogenic tumors. Since the late 1970s, the bacillus Calmette-Guerin vaccine has been used for intravesical instillation in nonmuscle invasive bladder cancer. Until the discovery of tyrosine kinase inhibitors in 2007, interleukin-2 and interferon alpha were the standard of care for metastatic renal-cell cancer. Checkpoint inhibitors for urothelial cancer and Nivolumab for metastatic renal-cell carcinoma are already approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Moreover, there are many checkpoint inhibitors in different phases of clinical development in the treatment of urological tumors or genitourinary cancers.[10,11]

Of note, anti-PD-1 agent Nivolumab has shown efficacy in head and neck cancers, which share squamous histology and HPV infection pathogenic characteristics with PeSCC. Among patients with platinum-refractory, recurrent

squamous cell carcinoma of the head and neck, treatment with Nivolumab resulted in longer overall survival than treatment with standard single-agent therapy and delayed time to deterioration of patient-reported quality-of-life outcomes. These data supported Nivolumab as a new standard-of-care option in this setting of patients.[12]

To our knowledge there are not yet active clinical trials regarding the treatment of patients with advanced PeSCC with anti-PD-1/PD-L1 immunotherapy. A relative clinical trial of Nivolumab combined with Ipilimumab for patients with advanced rare genitourinary tumors, has been announced but is not yet recruiting patients (NCT03333616).

In conclusion, in patients with metastatic PeSCC active immunotherapy combinations with an anti-PD-1/PD-L1 agent may be beneficial. Industry currently tends to not invest in treating rare diseases such as penile cancer. However, continued efforts should be made by independent investigators to contribute in advancing the treatment of such a devastating disease, given its high morbidity and mortality.

COMPLIANCE WITH ETHICAL STANDARDS

Informed consent was obtained from patient and local institutional review boards approved the therapy protocol. Permissions and approval for administrating treatment with Nivolumab were obtained from Scientific Committee for Off-Label Drug Use, Hellenic National Organization for Medicines. All procedures performed in studies involving human participants were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

CONFLICTS OF INTEREST/FINANCIAL DISCLOSURES

None reported. All authors have declared there are no financial conflicts of interest with regard to this work.

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