Tumor molecular profiling of responders and non-responders following pembrolizumab monotherapy in chemotherapy resistant advanced cervical cancer

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\textbf{ABSTRACT}

Optimal treatment for advanced cervical cancer after first line chemotherapy remains undefined. Immune checkpoint inhibition with pembrolizumab, a programmed cell death protein 1(PD-1) inhibitor, is under investigation. We analyzed the micro-environmental and molecular genetic profile of tumors from 4 patients with metastatic cervical cancer treated with off-label second-line pembrolizumab in an effort to identify predictive biomarkers. All patients received 2 mg/kg of pembrolizumab, 3-weekly until disease progression. Immunohistochemistry (IHC) for PD-1, PD-L1, CD3 and CD8, as well as next generation sequencing (NGS) for 50 cancer-related genes were performed on tumor samples. All patients tolerate treatment well with no discontinuation of treatment due to toxicity. One patient experienced dramatic and prolonged partial response, and remains stable on pembrolizumab with a progression free survival (PFS) of 21 months at the time of reporting of this series. Three patients experienced disease progression as best response. In the exceptional responder, there was no tumoral expression of PD-L1, however, combined positive score (CPS) for PD-L1 was ≥1% of stromal lymphocytes. All patients with response or clinical benefit had CPS for PD-L1 ≥1%. NGS revealed PIK3CA mutations in 3 tumors. Pembrolizumab is a promising therapeutic option in advanced cervical cancer. Further evaluation of biomarkers may guide optimal patient selection.

1. Introduction

Cervical cancer is the second most common cancer among women in less developed countries (Torre et al., 2015). The GOG 240 trial transformed the treatment of advanced cervical cancer by demonstrating improved survival with bevacizumab added to standard chemotherapy (Tewari et al., 2014). Optimal treatment after progression on anti-angiogenic therapy remains unclear.

PD-1/PD-L1 (Programmed cell death protein 1/Programmed death-ligand 1) inhibition may be a viable therapeutic strategy in cervical cancers. PD-L1 expression has been reported in 95% of cervical intraepithelial neoplasia (CIN) and 80% of cervical squamous cell carcinomas (Mezache et al., 2015). A large proportion of squamous cell carcinoma of the cervix, and nodal metastases, have been characterized to harbor high levels of PD-L1+ antigen-presenting cells (APCs) and FOXP3+ regulatory T cells (Tregs) (Heeren et al., 2015). The PD-1/PD-L1 interaction in human papilloma virus (HPV)-associated head and neck squamous cell cancer (HNSCC) has also been shown to create an “immune-privileged” site for initial viral infection, and subsequent adaptive immune resistance once tumors are established (Lyford-Pike et al., 2013). This provides rationale for therapeutic PD-1/PD-L1 blockade in HPV-associated tumors, such as cervical cancer.

In the phase Ib KEYNOTE-28 study, 24 patients with advanced cervical cancer were treated with pembrolizumab, a PD-1 inhibitor. All
patients had PD-L1 expression in ≥1% of tumor or stromal cells by immunohistochemistry (IHC). At a median of 11 months follow-up, the confirmed objective response rate (ORR) was 17%, with partial response (PR) seen in 4 of 24 patients (Frenel et al., 2017). The role of pembrolizumab is currently being further investigated in the phase II KEYNOTE-158 trial (NCT02628067). Hitherto, there remains a lack of published data on relevant biomarkers in cervical cancer patients who have responded to, or are resistant to pembrolizumab therapy.

We report on 4 patients with recurrent or metastatic cervical cancer, treated with off-label pembrolizumab, after progression on initial platinum-based chemotherapy. One patient was an exceptional responder. We performed in-depth microenvironment and molecular genetic profiling of their tumors. Three patients received radiotherapy upon progression on pembrolizumab, in an attempt to reverse resistance to PD-1 inhibition by induction of an abscopal response.

2. Materials and methods

2.1. Patient selection

Ethical approval for molecular analysis of patient tumor samples was obtained from the National Health Group Review Board (2014/00131). Clinical data and tumor samples from four patients with metastatic cervical cancer, treated with off-label second-line pembrolizumab, at our institution from June 2015 to January 2017, were retrospectively analyzed. Patient responses were evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria (version 1.1) with Computed Tomography (CT) or Fluorine-18 (F-18) fluorodeoxyglucose (FDG) positron emission tomography (PET) CT scans every 2 cycles. Progression-free survival (PFS) was defined as the interval from commencement of pembrolizumab until disease progression by RECIST v1.1.

2.2. Immunohistochemistry

IHC was performed using the Bond Polymer Refine Detection Reagent on the Bond-Max autostainer (Leica, Wetzlar, Germany). Antibodies used were 22C3 (pharmDx, Dako, Glostrup, Norway) at 1/100 dilution for PD-L1, NAT105 (Abcam, Cambridge, UK) at 1/100 for PD-L1, 144B (Dako, Glostrup, Norway) at 1/600 for CD8, and LN10 (Leica) at 1/50 for CD3. CD8 and CD3 are T-cell specific markers, staining of which was intended to better define tumoral and stromal lymphocyte density. Combined positive score (CPS) was calculated using the ratio of PD-L1 staining tumor and immune cells, to total viable tumor cells (Bellmunt et al., 2017).

2.3. Next generation sequencing

DNA was extracted from 3 sections (5 μm each) of formalin-fixed paraffin-embedded (FFPE) tumor samples using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany). Next generation sequencing (NGS) was performed using the Ampliseq Cancer Hotspot v2 Panel and the Ion Torrent Personal Genome Machine (Thermo Fisher Scientific, Waltham, MA) (Table S1). Variants with quality score > 200, in coding regions, non-synonymous and with minor allele frequency of < 5% in East Asian and South Asian populations in the 1000 Genomes Databases, were considered for report. The tumor from Patient 1 also underwent sequencing using the SmartGen NGS-467 assay (Precipio Diagnostics, New Haven, CT).

3. Results

3.1. Patient demographics

Patient demographics and clinical outcomes following pembrolizumab are summarized in Table 1. 2 patients (Patients 3 and 4) had upfront metastatic cancer and 2 (Patients 1 and 2) had relapsed disease. Patient 1 received tracheectomy for International Federation of Gynecology and Obstetrics (FIGO) stage IB1 cancer but relapsed 3 years after primary therapy, with widespread metastases. Patient 2 received chemo-radiation for FIGO stage IB2 cancer, with cisplatin (40 mg/m²) weekly concurrent with 50 Gy external beam radiotherapy in 28 fractions, followed by an extended field boost of 9 Gy in 5 fractions to low para-aortic lymph nodes, and triple channel brachytherapy. She completed 3 out of 4 planned cycles of adjuvant chemotherapy with carboplatin dosed at area under curve (AUC) 5 and paclitaxel 175 mg/m² 3-weekly. Residual FDG PET-avid disease in a single external iliac lymph node was surgically resected. She relapsed 6 months later with visceral and nodal metastases.

Patients 1, 2 and 3 received front-line cisplatin (50 mg/m²), paclitaxel (175 mg/m²) and bevacizumab (15 mg/m²) 3-weekly, without maintenance bevacizumab. Patient 4 developed G4 hypersensitivity to paclitaxel during cycle 1. She was switched to cisplatin (50 mg/m² on day 1), gemcitabine (1000 mg/m² on day 1 and 8), 3-weekly. All patients received off-label pembrolizumab 2 mg/kg 3-weekly, in the second-line.

3.2. Patient outcomes following pembrolizumab

PR was observed in 1 patient (Patient 3), after 3 cycles of pembrolizumab, which was durable, with PFS of 21 months, at the time of reporting of this series. PD was observed in 3 patients, after 1.5 months each, and confirmed with repeat scan after a further 4 weeks. Nonetheless, 2 patients (Patient 2 and 4) had continued clinical benefit after time of declared radiological PD. This manifested in reduced pain over a symptomatic nodal site (Patient 2), as well as weight gain of 10% body weight and improvement of performance status from 1 to 0 (Patient 4). Both patients opted to continue on pembrolizumab for a total duration of 3.5 months (Patient 2) and 7.5 months (Patient 4), respectively before symptomatic disease progression necessitating discontinuation of treatment.

3.3. Adverse events on pembrolizumab therapy

No G3 or higher toxicities, based on Common Terminology Criteria for Adverse Events version 4.0 (CTCAE v4.0), were encountered. Patients experienced G1 fatigue (4/4 patients), G1 rash (1/4 patients) and G1 hypersensitivity (1/4 patients). Patient 3 developed G2 anorexia, fatigue, weight loss and vomiting, 14 months after commencing pembrolizumab. She was diagnosed with G2 cortisol insufficiency. Pituitary imaging revealed a partial “empty sella” appearance. Her adrenocorticotropic hormone and cortisol levels were low, suggesting pembrolizumab-induced hypophysitis with partial hypopituitarism. She started on physiological doses of hydrocortisone which resolved her symptoms. She chose to continue on pembrolizumab. No dose reduction was implemented at re-introduction of pembrolizumab, and she remained well on hydrocortisone replacement.

3.4. Efficacy of radiation with pembrolizumab upon progression to induce an abscopal effect

Patients 1, 2 and 4 received radiotherapy after progression on pembrolizumab. Patients 1 and 4 resumed pembrolizumab after radiotherapy (Table 1).

Patient 1 progressed after 2 cycles of pembrolizumab with worsening vertebral metastases causing spinal instability. She underwent spinal stabilization surgery followed by third-line platinum based chemotherapy. On PD after this line of treatment, she developed symptomatic liver metastases with pain and transaminitis. Two fractions of stereotactic beam radiotherapy, 14 Gy each, were administered to the 2 largest hepatic lesions. Pembrolizumab 2 mg/kg was re-challenged 2 days after radiotherapy completion, with the goal of inducing an
Table 1
Patient and tumor characteristics, treatment and outcome.

|                      | Patient 1 | Patient 2 | Patient 3 | Patient 4 |
|----------------------|-----------|-----------|-----------|-----------|
| Age                  | 37        | 31        | 50        | 49        |
| Ethnicity            | Caucasian | Chinese   | Arabic    | Chinese   |
| Histology            | SCC       | ADC       | SCC       | SCC       |
| First-line treatment received | TP + Bev  | TP + Bev  | TP + Bev  | GP        |
| Best response to first-line treatment | PR       | PD       | PR       | SD        |
| PFS on first-line treatment (months) | 6        | 2        | 8        | 6         |
| Dose of Pem given (mg/3-weekly) | 100      | 100      | 150       | 100       |
| Best Response to second-line Pem | PD       | PD       | PR (durable) | PD       |
| Clinical benefit from Pem | No       | Yes      | Yes       | Yes       |
| PFS on Pem (months)   | 1.5       | 1.5      | 21+ (ongoing) | 1.5       |
| RT Site and dosage/fraction | T7 to S2 (27Gy/3#) | Mediastinal LN (36Gy/12#) | No        | Pelvis (40Gy/16#) |
| Rechallenge Pem after RT | Yes     | No       | No        | Yes       |
| Response to Pem after RT | No       | No       | No        | Yes       |
| Site of tumor profiled p16 expression | Liver | Peritoneal deposit | Para-aortic LN | Primary |
| CD8 expression       | Diffusely positive | Diffusely positive | Diffusely positive | Negative |
| CD3 expression       | Scattered intratumoral and peritumoral lymphocyte expression | < 10% peritumoral lymphocyte expression | Scattered intratumoral lymphocyte expression | Scattered intratumoral lymphocyte expression |
| PD-1 expression      | No expression | No expression | No expression | No expression |
| TPS for PD-L1        | No expression | 40% tumoral expression | No expression | 10% tumoral expression |
| CPS for PD-L1        | 0         | 50        | 1         | 1         |
| NGS (AmpliSeq)       | Pik3ca (E545K) | PTEN (D588fsTer41) | ERbb4 (E612W) | Pik3ca (E542K) |
| NGS (SmartGen)       | BRCA1 (E143Ter) | not performed | Pik3ca (E545K) | Cdh1 (D402H) |

SCC: Squamous cell carcinoma; ADC: Adenocarcinoma; TP + Bev: D1 Cisplatin 50 mg/m², Paclitaxel 175 mg/m², Bevacizumab 15 mg/kg (q 21 days); Pem: D1 Pembrolizumab 2 mg/kg (q 21 days); GP: D1 Cisplatin 50 mg/m², D1 & D8 Gemcitabine 1000 mg/m² (q 21 days); RT: Radiotherapy; IHC: Immunohistochemistry; NGS: Next generation sequencing; +: Ongoing response at time of review; Met: Metastasis; Pri: Primary tumor; LN: lymph node; TPS: Tumor Proportion Score; CPS: Combined Positive Score.

Fig. 1. PD-L1 expression.
1(a): Patient 1, tumor proportion score (TPS) 0%; (b): Patient 2, TPS 40%; (c): Patient 3, TPS 0%; (d): Patient 4, TPS 2%.
abscopal effect. However, further hepatic progression was noted after 2 cycles of pembrolizumab re-challenge.

Prior to pembrolizumab, Patient 2 had relapsed disease with lymphadenopathy of the left supravacular fossa (SCF), thoracic and retroperitoneal lymph nodes associated with neck and back pain. Following her first cycle of pembrolizumab, she experienced clinical shrinkage of her left SCF node and symptomatic improvement in her neck and back pain with reduction of her tumor markers. Unfortunately, by the end of the second cycle, restaging scans showed PD by RECIST v1.1. She was continued on pembrolizumab to a total of 4 cycles (3 months), during which she developed dysphagia from enlarging mediastinal lymphadenopathy followed by pain over the re-enlarging left SCF node. External beam radiotherapy (36Gy in 12 fractions) to both symptomatic nodal sites. Pembrolizumab was discontinued.

Patient 4 developed radiological PD as per RECIST v1.1 after 1.5 months of treatment, yet continued to have marked improvement of symptoms in terms of weight gain and performance status. She opted to continue pembrolizumab. After 10 cycles (7.5 months) of pembrolizumab, she eventually developed symptomatic progression of the disease in her liver associated with pain. Pembrolizumab therapy was interrupted for external beam radiotherapy to the liver (16 Gy in 2 fractions), and was resumed for 2 further cycles, commencing at 2 days post-completion of radiotherapy. No response was seen in the non-irradiated visceral metastases, however, shrinkage of the non-irradiated retroperitoneal and inguinal lymph node metastases was noted after the 2 further cycles of pembrolizumab.

3.5. PD-L1 and PD-1 assessment in tumors

Tumor samples were analyzed for PD-1, PD-L1 (tumor proportion score (TPS)) and combined positive score (CPS)), CD3 and CD8 expression (Table 1 and Fig. 1). CPS of ≥1 was observed in patients who achieved symptomatic improvement or PR on pembrolizumab (Patients 2, 3 and 4). Rapid symptomatic progression and disease progression were seen in Patient 1, whose CPS was 0. Focal PD-1 expression in peritumoral lymphocytes was seen in the tumor of both Patients 3 and 4, who had long term (>6 month) symptom stabilization (Patient 4) and durable PR (Patient 3).

3.6. Tumor mutational analysis

Sites sampled for NGS and tumor mutations observed, are described in Table 1. PIK3CA mutations were observed in 3 out of 4 patients, namely E545K, E542K and H1047Y. All PIK3CA mutations were found to be pathogenic with a gain-of-function mutation effect. The SmartGen NGS-467 assay for Patient 1 detected the PIK3CA (E545K) mutation, but also a BRCAl (p.E143X) mutation from its extended scope of interrogated genes (Table S2).

4. Discussion

Early phase trials using immune checkpoint inhibitors are currently on-going in cohorts that include cervical cancer patients (NCT02628067, NCT01975831, NCT01711515). Nonetheless, reports of durable responses and good-tolerability seen in KEYNOTE-028 (NCT02054806) have prompted off-label second line use of pembrolizumab in patients with metastatic cervical cancer at our institution and elsewhere. Although the current FDA approved dose for pembrolizumab in most tumor types is a 2 mg/kg 3-weekly dose, the 2 mg/kg 3-weekly dose was chosen at that time, as it had been shown to have similar anti-cancer activity when compared with higher doses in early phase studies (Robert et al., 2014; Garon et al., 2015), and was less costly for patients. Further analyses have now revealed similar pharmacokinetic variability for the 2 mg/kg and fixed 200 mg 3-weekly doses (Freshwater et al., 2017), with similar exposure distribution. This is because pembrolizumab binding to PD-1 receptors on T cells does not depend on direct engagement of the drug with tumor cells (Freshwater et al., 2017), resulting in no difference between dose-response and exposure-response. This equivalent efficacy when compared even more dose-intense regimens, such as 10 mg/kg 3-weekly (Garon et al., 2015), further reinforced that 2 mg/kg 3-weekly is likely able to achieve near-maximal clinical efficacy, similar to what was seen on pre-clinical assays (Lindauer et al., 2017).

PD-L1 tumoral expression and CPS have been suggested as potential biomarkers for PD-1 inhibitors, in other advanced cancers. CPS has been reported as being associated with higher ORR when positive (≥1) in HNSCC (Bauml et al., 2017). Of note, pembrolizumab was recently granted accelerated approval as monotherapy in previously treated gastric or gastroesophageal junction adenocarcinomas, with PD-L1 CPS ≥ 1 (Fuchs et al., 2017). In our series, patients who achieved disease response, stabilization or clinical benefit with pembrolizumab (Patients 2, 3, 4) all showed PD-L1 CPS ≥ 1. Of note, CPS was highest in Patient 2, who only experienced a short period of symptomatic improvement on pembrolizumab, compared to Patient 3, the exceptional responder, who had a lower CPS of only 1.

Attempts at inducing an ‘abscopal’ effect by integrating short-course radiotherapy to symptomatic sites, were made in 3 patients. This, referring to an out-of-field effect of immunogenic cell death seen in distant metastases, after a course of local irradiation, is thought to be consequent to an activation of the immune system by radiotherapy (Demaria and Formenti, 2012; Demaria et al., 2006), however its mechanism is not yet completely understood (Formenti and Demaria, 2013). Preclinical studies demonstrate that the combination of radiotherapy and PD-1/PD1 therapy can activate cytotoxic T-cells, and reduce myeloid-derived suppressor cells, promoting cancer recognition and tumor cell kill, even outside of the radiation field (Doveili et al., 2016; Deng et al., 2014; Andrew et al., 2015). Radiation exposure is thought to generate increased danger signals including IL-1β, TNFα, and other inducers of dendritic cell maturation such as prostaglandin E. Antigen-presenting cells then take up tumor-associated antigens released by the irradiated cells dying by necrosis, and are activated (Demaria and Formenti, 2013). Few studies have reported remarkable and durable regression of non-irradiated metastases after conventional radiotherapy, with and without immunotherapy, in several advanced cancers (Siva et al., 2016; Postow et al., 2012; Golden et al., 2015; Golden et al., 2013). Successful case studies, or series of an appreciable abscopal effect, have yet to be published in the context of advanced cervical cancer treated with PD-1 inhibition and radiation. In our series, only Patient 4 experienced shrinkage of disease outside the irradiated field when re-challenged with pembrolizumab post radiotherapy. However, this was limited to nodal disease, with no response in other sites, and overall clinical and radiological PD. This phenomenon may therefore only be relevant in tumors with specific immune micro-environment features.

Somatic mutations in PIK3CA (Patient 1, 3 and 4) or PTEN (Patient 2) were identified in our patients’ tumors. Genomic alterations in PI3K–MAPK and TGFβ signaling pathways are well described in cervical cancers, however their role in tumor progression is not fully defined (Lee et al., 2015). In melanoma models, loss of PTEN in tumor cells has been shown to inhibit T-cell mediated tumor kill and reduce T-cell trafficking into tumors, which correlates with inferior outcomes to PD-1 inhibitor therapy (Peng et al., 2016). Durable PR was seen in Patient 3, whose tumor had a PIK3CA mutation, suggesting that this may not be the case in cervical cancer.

Much interest has surrounded the effect of homologous recombination repair pathway defects on intrinsic tumor immunogenicity. Tumors with DNA repair defects are thought to be associated with a high expression of neoantigens, associated with increased immune stimulation, as evidenced by higher levels of tumor-infiltrating lymphocytes (TILs) in these tumors (Le et al., 2015). Patient 1 was found to have a pathogenic germline BRCAl1 mutation (p.E143X).
but this did not seem to affect response to pembrolizumab, as she had rapid PD after 2 cycles and no clinical benefit. Patient 3 was found to have numerically more mutations on NGS compared to the other patients in this series, this itself could indicate higher mutational burden and thus increased susceptibility to immune checkpoint inhibition.

Limitations of our study include the small dataset, and limited gene panel for molecular profiling. To our knowledge, the report describes the first comprehensive assessment of tumor microenvironment and genomic biomarkers associated with PD-1 inhibitor therapy in patients with metastatic cervical cancer. Based on our small dataset, patients with PD-L1 CPS ≥ 1 showed symptomatic benefit with pembrolizumab. The magnitude of CPS did not appear to correlate with duration of symptomatic benefit or response. Further biomarker data from larger clinical studies of PD-1 inhibitors in cervical cancer are currently awaited.

5. Conclusion

In summary, PD-1 inhibitors appear to be a viable option and are well tolerated with preliminary evidence of efficacy in selected patients. As several clinical trials are underway, further evaluation of biomarkers, including PD-L1 CPS, may help to guide optimal selection of immunotherapy approaches for women with metastatic, persistent or recurrent cervical cancer.

Conflict of interest statement

Dr David SP Tan declares honoraria from MSD.

All other authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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