First-in-human Intravesical Delivery of Pembrolizumab Identifies Immune Activation in Bladder Cancer Unresponsive to Bacillus Calmette-Guérin

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Abstract

Background: Intravenous immune checkpoint inhibition is an effective anticancer strategy for bacillus Calmette-Guérin (BCG)-unresponsive non–muscle-invasive bladder cancer (NMIBC) but may be associated with greater systemic toxicity compared with localized therapies.

Objective: We assessed the safety and antitumor activity of intravesical pembrolizumab combined with BCG.

Design, setting, and participants: A 3 + 3 phase 1 trial of pembrolizumab + BCG was conducted in patients with BCG-unresponsive NMIBC (NCT02808143).

Intervention: Pembrolizumab was given intravesically (1–5 mg/kg for 2 h) beginning 2 weeks prior to BCG induction until recurrence. Urine profiling during treatment and spatial transcriptomic profiling of pre- and post-treatment tumors were conducted to identify biomarkers that correlated with response.

Outcome measurements and statistical analysis: Safety and tolerability of immune checkpoint inhibition were assessed, and Kaplan-Meier survival analysis was performed.

Results and limitations: Nine patients completed therapy. Median follow-up was 35 months for five patients still alive at the end of the trial. The trial was closed due to the COVID-19 pandemic. Grade 1–2 urinary symptoms were common. The maximum tolerated dose was not reached; however, one dose-limiting toxicity was reported (grade 2 diarrhea) in the only patient who reached 52 weeks without recurrence. One death occurred from myasthenia gravis that was deemed potentially related to treatment. The 6-mo and 1-yr recurrence-free rates were 67% (95% confidence interval [CI]: 42–100%) and 22% (95% CI: 6.5–75%), respectively. Pembrolizumab was detected in the urine

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and not in blood. CD4+ T cells were significantly increased in the urine after treatment, and a transcriptomic analysis identified decreased expression of T-cell exhaustion markers in late recurrences.

Conclusions: We demonstrate that intravesical pembrolizumab is safe, feasible, and capable of eliciting strong immune responses in a clinical setting and should be investigated further.

Patient summary: Direct application of pembrolizumab to the bladder is a promising alternative for non–muscle-invasive bladder cancer unresponsive to Bacillus Calmette-Guérin and should be investigated further.

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1. Introduction

Treatment for non–muscle-invasive bladder cancer (NMIBC) is endoscopic resection followed by intravesical adjuvant chemotherapy or bacillus Calmette-Guérin (BCG) [1]. Non-responders to BCG have limited options. In 2021, the first intravenous anti-PD1 checkpoint immunotherapy (CPI) was approved for patients with BCG-unresponsive NMIBC with carcinoma in situ [2]. Although the 3-mo response was 41% (39/96), the median duration of a complete response (CR) was 16 mo (95% confidence interval [CI]: 7–36), with 19% (18/96) CR at 12 mo. While pembrolizumab was well tolerated, 66% of patients reported an adverse event (AE), with a total of 11 serious AEs (SAEs) in eight patients. Hence, even though some patients responded to pembrolizumab, >80% did not respond and two of three reported AEs.

We hypothesized that changing the route of delivery could limit toxicity. For >40 years, urologists have administered medication into the bladder to treat bladder cancer [3,4]. Recently, several trials have focused on intravesical administration of immune-modulating agents to generate a localized immune response [5]. To date, activity and safety profile of CPIs administered as an intravesical therapy have not been investigated. Recent preclinical studies in mice suggest an increase in lymphocytes in the bladder after intravesical CPI antibodies [6]. Herein, we report safety, toxicity, and clinical response from the first-in-human clinical trial of intravesical administration of an immune checkpoint inhibitor.

2. Patients and methods

2.1. Study design and participants

NCT02808143 was a phase 1 dose-escalation trial with a 3 + 3 design using increasing doses of intravesical pembrolizumab with BCG in patients unresponsive to BCG. All patients were recruited from one institution. The trial was approved by Northwestern University IRB (STU00202754) and the Robert H. Lurie Comprehensive Cancer Center Scientific Review Committee and Data Safety and Monitoring Committee. Patients had a history of high-grade bladder cancer (Ta, T1, or Tis) and were treated with at least seven doses of BCG (five during induction and two during maintenance). Patients were also eligible if they were BCG-unresponsive and had been treated with at least three doses of a salvage regimen (such as gemcitabine and/or docetaxel). Any patient with an invasion (T1) must have had imaging within 60 days, and all participants had to have a Eastern Cooperative Oncology Group performance of 0 or 1. The inclusion and exclusion criteria are listed in the protocol available on request.

2.2. Study procedures and dose-escalation scheme

After enrollment, patients received a single intravesical dose of pembrolizumab at the specified dose level (1 or 2 mg/kg) at week –2 prior to induction (Fig. 1B). Participants were then treated with BCG (TICE; 50 mg, weeks 0–5) and intravesical pembrolizumab (weeks 0, 2, and 4). After induction, participants received only intravesical pembrolizumab during maintenance, first every 2 weeks until week 17 and then every 4 weeks for the remainder of the year. Dose-limiting toxicities (DLTs) were defined as SAEs from week –2 through week 5 and defined by Common Terminology Criteria for Adverse Events v4.03. Additional details can be found in the Supplementary material.

2.3. Outcome measures

At each visit, patients underwent an evaluation and physical examination to identify any AEs with endoscopic evaluation at week 17 and every 8 weeks until the completion of the trial at week 52 (last cystoscopy on week 49). Urine was analyzed for cytopathology and any concerning lesion was biopsied. Any high-grade recurrence, whether in the bladder or throughout the urinary tract, was considered a recurrence and treatment was halted. After the last dose of trial therapy, patients were followed clinically for disease recurrence and survival every 3 mo (±30 d) during years 1 and 2.

2.4. Statistics

This is a phase 1 dose-escalation study using the 3 + 3 design with four doses. All analyses were conducted using R version 4.0.3 or GraphPad Prism version 9.3.1. Wilcoxon signed rank test was used for paired sample comparisons (eg, from before to after comparison). For comparing independent groups, the Kruskal-Wallis test was used to generate p values. For repeated measures across different time points, a mixed-effect model was used to generate p values. Recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS) were defined as time from the start of pre-induction until the corresponding event. Patients were censored for PFS and OS on the date of last cystoscopy or imaging without observed progression or the date the patient was last known to be alive. Survival estimates were obtained using the method of Kaplan-Meier, and groups were compared using the log-rank test.

Detailed methods for urine pK, urine flow cytometry, serum pK, urine cytokine analysis, GeoMx digital spatial profiling, and PD-L1 immunohistochemistry are included in the Supplementary material.
3. Results

3.1. Clinical trial design

Eleven patients were screened, and nine were enrolled between June 2016 and May 2020 when the trial was closed due to the coronavirus disease 2019 (COVID-19) pandemic (Supplementary Fig. 1A). Patients were followed up through May 2021. Patient demographics are described in Table 1. All patients received a preinduction dose at week –2 (Fig. 1A) followed by BCG at weeks 0–5 along with intravesical pembrolizumab at weeks 0, 2, and 4. After induction, participants received intravesical pembrolizumab every 2 weeks until week 17 (first cystoscopy), and then every 4 weeks for the remainder of the year.

3.2. Toxicity

There were 21 grade 1–2 AEs related to BCG and/or pembrolizumab and one grade 5 AE related to pembrolizumab (Table 2). Nearly all BCG-related AEs were bladder related, with the most common being gross hematuria (five cases; Table 2). AEs are described in Table 2. The trial was closed early due to the COVID-19 outbreak. Three patients were treated at a starting dose of pembrolizumab 1 mg/kg (cohort 1), and six were treated at 2 mg/kg (cohort 2). One
DLT of grade 2, diarrhea lasting 21 d, was observed during treatment in cohort 2. One patient died due to treatment-related myasthenia gravis, an autoimmune disorder. The patient presented with vision changes and an elevated anti-ACHR antibody level. This was originally reported 117 d after the last dose of pembrolizumab. The patient was treated with pyridostigmine, prednisone, and intravenous immunoglobulin and expired 10 mo after symptom onset. All patients in both dose cohorts experienced grade 1–2 events (3/3 and 6/6 patients) related to BCG.

### 3.3. Clinical response data

The response to pembrolizumab and BCG was evaluated by cystoscopy, urine cytology, and bladder biopsy when indicated. The median follow-up time among five patients who were alive at the end of the study was 35 months (interquartile range: 26–36). All patients recurred with RFS rates of 100%, 67% (95% CI: 42–100%), and 22% (95% CI: 7–75%) at 3, 6, and 12 months, respectively (Fig. 1B and Supplementary Fig. 1B). The median RFS was 6.2 months (95% CI: 5–not available [NA]). No differences in RFS rates was observed between the two cohorts (log-rank test, \(p = 0.6\); Fig. 1C). The patient with the longest time to recurrence of 31 months was also the only participant who experienced a DLT.

Progression occurred in five patients with median PFS of 36 months (95% CI: 10–not reached). The PFS rates at 6 and 12 months were 100% and 56%, respectively (95% CI: 31–100%; Fig. 1D and Supplementary Fig. 1B). There were no significant differences in recurrence and progression rates when the cohort was stratified by carcinoma in situ (CIS; Supplementary Fig. 1B and 1C). Progression to locally advanced cancer (T2+N\(^\text{any}\), TxN+, or M+) occurred in four

<table>
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BCG = Bacillus Calmette-Guérin; CIS = Carcinoma In Situ; ECOG = Eastern Cooperative Oncology Group.
of nine patients, with recurrence in six of nine patients outside of the treated bladder—upper tract urothelial (two), lung (one), prostate (two), and pelvis (one) (Supplementary Table 5). Death occurred in four of nine patients (Fig. 1E and Supplementary Fig. 1D). Major cancer surgeries after enrollment included radical cystectomy (two patients), nephroureterectomy (one), and distal ureterectomy with partial cystectomy (one).

**3.4. Response during therapy: urine analytes**

To determine whether pembrolizumab was confined to the bladder, we evaluated plasma and urine levels of pembrolizumab for cohort 1, and serum levels in the patient with a DLT and a grade 5 SAE. We found no detectable levels of pembrolizumab in the blood (serum or plasma) in any patient. We were able to detect pembrolizumab in the urine samples collected at weeks –2 and 4. No detectable levels of pembrolizumab were found in the urine sample collected prior to treatment at week 4 (Supplementary Fig. 2A).

To understand local immune changes that occurred after intravesical pembrolizumab administration, we prospectively evaluated the urine of patients collected during the trial (Fig. 1A) [7]. To identify the potential mechanisms of a response, we compared patients with early recurrence (n = 5, median months to recurrence: 5.3, 95% CI: 4.9–NA) to late recurrence (n = 4, median: 12 months, 95% CI: 10–NA).

At baseline, the patient who developed myasthenia gravis had the highest level of CD45+ immune cells with an increase of 8.7-fold over the median of other patients at baseline (before treatment, week –2; Fig. 2A).

Next, we investigated whether a single dose of pembrolizumab could alter local immune environment by comparing the urine collected at baseline (before treatment, week –2) with that collected 2 weeks after the first dose of intravesical pembrolizumab (before treatment, week 0). Although most of the pretreatment urine collected at baseline had a large concentration of granulocytes, we observed a significant decrease in granulocytes (median: 41%, 95% CI: 0–60%). Wilcoxon signed rank test, p = 0.023, and a significant increase in CD4+ (median: 18%, 95% CI: 0.2–33%, p = 0.023) and CD8+ T cells (median: 12%, 95% CI: 0–14.2, p = 0.015) after a single dose of pembrolizumab (Fig. 2B). Since this measurement was performed 2 weeks later, alterations in immune cell populations reflect at least transient changes to the microenvironment after a single intravesical dose of pembrolizumab.

To identify immune changes that occurred with BCG and pembrolizumab, we compared urinary immune profiles between early and late recurrences at each week during the 6 weeks of induction. The most striking differences in immune populations were observed for time points when the patients received BCG and pembrolizumab (weeks 0, 2, and 4) relative to BCG alone (weeks 1, 3, and 5). Relative to early recurrences, late recurring populations had significantly lower fractions of granulocytes and a higher fraction of lymphocytes, in particular higher CD4+ and CD8+ T cells, at weeks 0 and 2 (Fig. 2C and Supplementary Table 6).

By urine cytokine profiling, we detect increased levels of BCG-regulated inflammatory cytokines (tumor necrosis factor [TNF]-alpha and interleukin [IL]-8) [8,9]; chemokines such as MIP-1 beta and monocyte chemoattractant (MCP)-1 increased from week –2 to 5 during induction treatment (Supplementary Fig. 2B).

Collectively, a multiomic urine analysis identified immune recruitment to the bladder following exposure to intravesical BCG and pembrolizumab.

**3.5. Spatial transcriptomic analysis**

We sought to understand how intravesical pembrolizumab and BCG affected bladder tumors and investigate the mechanisms of resistance. Given the technical limitations in profiling CIS by bulk RNA-seq or macrodissection, we performed transcriptomic analysis of pre- and post-treatment tumors using NanoString’s digital spatial profiling technology (GeoMx DSP, NanoString, Seattle, WA, USA) (Fig. 1A) [10]. Using DNA, pan-cytokeratin, and CD68 and CD3 as protein markers for the tumor and tumor microenvironment (TME), we evaluated 151 areas of interest (AOIs) collectively from eight patients, 86 from post-treatment and 65 from pre-treatment tumors. A principal component analysis confirmed discrete clustering of PanCK+ (tumor) and PanCK– (TME) AOIs (Fig. 3A). We first evaluated bladder cancer subtypes in PanCK+ AOIs. While one tumor had features of a basal/squamous tumor, the rest expressed only luminal markers [11]. We compared AOIs from multiple regions, and found little intratumoral spatial heterogeneity in tumor subtype and no changes in subtype after treatment (Fig. 3B).

Next, we compared the transcriptomic profile of pre- and post-treatment tumor epithelium (PanCK+) using a gene-set enrichment analysis and found significant enrichment of inflammatory pathways in PanCK+ post-treatment tumor AOIs (Fig. 3C). Pretreatment tumor AOIs expressed minimal inflammatory gene expression programs, with the exception of proinflammatory TNF signaling, suggestive of prior BCG treatment. Post-treatment tumors depicted an inflamed phenotype with enrichment of pathways involved in immune regulation (inflammation and antigen presentation, and TGF-β signaling) and cytokine signaling (IL-6, IL-2, IFNA, CXCR4, TLR, and IL signaling).

Next, we evaluated changes in the TME (PanCK–), comparing AOIs of the same patient before and after therapy. We found a significant increase in ImmuneScore (median increase: 48, 95% CI: 12–104, Wilcoxon signed rank test, p = 0.031; Fig. 3D) in post-treatment TMEs. In particular, we found a significant increase in plasma (median difference: 6.5, 95% CI: 4.3–9.2), natural killer (NK; median difference: 3, 95% CI: 1.4–4.2), CD8+ T (median difference: 6.9, 95% CI: 4.5–9.9), and Treg (median difference: 7.0, 95% CI: 2.2–8.1) cells in post-treatment samples relative to pretreatment samples (Fig. 3E). This increase was most pronounced in the patient who developed myasthenia gravis (patient 104; Supplementary Fig. 3A and 3B).

To identify the mechanisms of resistance or response to intravesical pembrolizumab and BCG, we compared spatial profiling of pretreatment tumor and TME from early and
late recurrences. Tumor epithelium AOs from early recurrences showed an inflamed phenotype with upregulation of TNF-mediated inflammatory signaling and MHC class II signaling. In contrast, late recurrences were enriched for pathways involved in cell cycle regulation, DNA damage response, and innate immune pathways (Fig. 4A).

Next, we evaluated the pretreatment TME of early and late recurrent tumors. Early recurrent TME was enriched in stress response pathways, stromal signatures, and angiogenesis with a higher stromal score and significantly elevated levels of PDGFRB (Fig. 4B and Supplementary Fig. 3C). In contrast, late recurring TME had an enrichment of pathways associated with adaptive immune system processes (T-cell receptor and B-cell receptor signaling), cytokine signaling (IL-2 and NF-KB pathways; Fig. 4B) along with a significantly higher ImmuneScore (median difference: 26, 95% CI: 12–95; Supplementary Fig. 3D).

In particular, we find elevated CD8$^+$ T (median difference: 12, 95% CI: 6–19), Treg (median difference: 9.2, 95% CI: 2.3–11), B (median difference: 9.9, 95% CI: 3.5–36), plasma (median difference: 4.8, 95% CI: 1–5.5), and NK (median difference: 2.2, 95% CI: 0.18–5.2) cells in late recurring tumors relative to early recurrences (before treatment; Fig. 4C). To characterize the activation state of an immune response, we compared immune cell counts and exhaustion score. Late recurrent tumors had a large significant increase in immune cells in the TME, and a significant reduction in exhaustion markers from before to after treatment (Fig. 4D).

Overall, this is the first direct comparison of the heterogeneity of tumor and TME in NMIBC and BCG-unresponsive NMIBC by spatial profiling. Our data indicate that differences in clinical endpoints of BCG-unresponsive tumors may be secondary to distinct pathways of the tumor/TME, with responsive tumors showing greater T-cell infiltration with less exhaustion, while resistant tumors have increased neutrophils and a stroma-rich microenvironment (Supplementary Fig. 3E).

4. Discussion

Although CPI has revolutionized the treatment of solid tumors, toxicity of CPI and its efficacy in early-stage NMIBC require further investigation for broader use. In this trial, we attempted to mitigate the toxicity of CPI by direct administration of pembrolizumab into the bladder, potentially avoiding systemic toxicity. We combined pembrolizumab with BCG with the intent to both increase immune recruitment and decrease exhaustion. We had initially hoped to deliver higher doses of pembrolizumab; however, our trial was discontinued at the start of the COVID-19 pandemic.

We did not detect pembrolizumab in the serum or plasma in all five patients tested; however, two patients experienced toxicity during treatment, and both AEs occurred at the higher dose of pembrolizumab tested—2 mg/kg. While we cannot conclude that lower dosing of pembrolizumab is safe, higher doses may not be needed...
Modulating the immune response. We observed increased lymphocyte recruitment, suggestive of immune modulation 2 weeks after a single dose of intravesical pembrolizumab. Similar to other immunotherapies, we find a correlation between an inflammatory response and the durability of a clinical response. The patient who had the longest response to therapy, >12 months without high grade recurrence, was also the patient who experienced a DLT during induction. Except for two patients, intravesical pembrolizumab was well tolerated with only grade 1–2 AEs that were mostly urinary related. Thus, intravesical delivery of CPI is a viable strategy for patients with BCG-unresponsive NMIBC, merit¬ing further investigation of dose, delivery, and patient selection.

Evaluation of tissue and blood from the one patient with a grade 5 autoimmune AE identified possible mechanisms of systemic immune activation from intravesical therapy. This patient had the highest number of CD45+ cells in the urine prior to treatment initiation, and this number increased further during the course of treatment. Although this resulted in a longer delay to recurrence, the enhanced immune activation from intravesical therapy may have hastened progression of myasthenia gravis. While BCG and advanced age have been associated with myasthenia gravis, only a few case reports have described myasthenia gravis associated with intravenous checkpoint therapy [12,13].

There are several limitations of our study. Our sample size was limited to nine patients due to premature closure of the trial. Although we attempt to describe our findings for pre- and post-treatment samples, validation in larger cohorts is necessary. The rate of extravesical recurrence was greater than expected and may reflect the limited number of treatment options available for patients recruited in this study.

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5. Conclusions

Here, we demonstrate that intravesical pembrolizumab is a safe and feasible alternative in patients with BCG-unresponsive NMIBC. We attempted to decrease the toxicity of anti-PD1 therapy by intravesical delivery; however, two of nine patients had a DLT or an SAE.

We are encouraged by the broad range of immune responses observed in the urine collected during trial and in the tumor tissue at the end of the trial. Changes in the immune niche are detectable in the urine as early as after one dose of pembrolizumab. Nevertheless, as evident by analysis of recurrences observed in our trial, duration of antitumor immunity is dependent on pretreatment tumor composition, and future trials should explore combination therapies and careful patient selection to improve efficacy.

Overall, intravesical administration of pembrolizumab is a promising therapeutic modality, capable of generating long-lasting antitumor immunity, and should be investigated further.

Author contributions: Joshua J. Meeks had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Meeks, Kuzel, Svatek.

Acquisition of data: Meghani, Cooley, Choy.

Analysis and interpretation of data: All authors.

Drafting of the manuscript: Meghani, Meeks, Cooley, Choy.

Critical revision of the manuscript for important intellectual content: All authors.

Statistical analysis: Meghani, Munir, Kocherginsky.

Obtaining funding: Meeks.

Administrative, technical, or material support: Meghani, Choy, Swaminathan, Munir.

Supervision: Meeks, Kocherginsky.

Other: None.

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Peer Review Summary

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References