Intravesical rAd-IFN alpha/Syn3 for Patients With High-Grade, Bacillus Calmette-Guerin-Refractory or Relapsed Non-Muscle-Invasive Bladder Cancer: A Phase II Randomized Study

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ABSTRACT

Purpose
Many patients with high-risk non–muscle-invasive bladder cancer (NMIBC) are either refractory to bacillus Calmette-Guerin (BCG) treatment or may experience disease relapse. We assessed the efficacy and safety of recombinant adenovirus interferon alfa with Syn3 (rAd–IFNα/Syn3), a replication-deficient recombinant adenovirus gene transfer vector, for patients with high-grade (HG) BCG-refractory or relapsed NMIBC.

Methods
In this open-label, multicenter (n= 13), parallel-arm, phase II study (ClinicalTrials.gov identifier: NCT01687244), 43 patients with HG BCG-refractory or relapsed NMIBC received intravesical rAd–IFNα/Syn3 (randomly assigned 1:1 to 1 x 10^11 viral particles (vp)/mL or 3 x 10^11 vp/mL). Patients who responded at months 3, 6, and 9 were retreated at months 4, 7, and 10. The primary end point was 12-month HG recurrence-free survival (RFS). All patients who received at least one dose were included in efficacy and safety analyses.

Results
Forty patients received rAd–IFNα/Syn3 (1 x 10^11 vp/mL, n = 21; 3 x 10^11 vp/mL, n = 19) between November 5, 2012, and April 8, 2015. Fourteen patients (35.0%; 90% CI, 22.6% to 49.2%) remained free of HG recurrence 12 months after initial treatment. Comparable 12-month HG RFS was noted for both doses. Of these 14 patients, two experienced recurrence at 21 and 28 months, respectively, after treatment initiation, and one died as a result of an upper tract tumor at 17 months without a recurrence. rAd–IFNα/Syn3 was well tolerated; no grade four or five adverse events (AEs) occurred, and no patient discontinued treatment because of an adverse event. The most frequently reported drug-related AEs were micturition urgency (n = 16; 40%), dysuria (n = 16; 40%), fatigue (n = 13; 32.5%), pollakiuria (n = 11; 28%), and hematuria and nocturia (n = 10 each; 25%).

Conclusion
rAd–IFNα/Syn3 was well tolerated. It demonstrated promising efficacy for patients with HG NMIBC after BCG therapy who were unable or unwilling to undergo radical cystectomy.

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INTRODUCTION

Non–muscle-invasive bladder cancer (NMIBC) represents the most common disease state for patients with newly diagnosed bladder cancer. Those with high-grade (HG) tumors are at significant risk for both recurrence and progression. Bacillus Calmette-Guerin (BCG) represents the current preferred management. Nonetheless, approximately 30% of patients will not respond to BCG; among those who demonstrate an initial response, more than 50% will experience recurrence and progression during long-term follow-up.

The optimal management of patients with persistent or recurrent tumor after BCG remains controversial. Although radical cystectomy provides cancer eradication, many patients are elderly, have significant comorbidities with an attendant diminished performance status, and often are unwilling to undergo radical extirpative...
surgery. Nonextirpative treatment options are available, but studies to date have included relatively small patient numbers and used varied definitions of treatment success.\textsuperscript{8,10-16} Indeed, the US Food and Drug Administration (FDA) and genitourinary oncology community agree that scant progress has been made in the management of this disease since the initial approval of BCG.\textsuperscript{17-19} Thus an effective alternative to radical cystectomy for patients with disease recurrence after BCG treatment remains an important unmet clinical need.\textsuperscript{17}

Recombinant intravesical interferon alfa-2b protein (IFN\textalpha\textsubscript{-}2b; Intron A; Merck, Kenilworth, NJ) demonstrated promising initial clinical results in NMIBC.\textsuperscript{20,21} Intravesical IFN\textalpha\textsubscript{-}2b gene delivery offers a novel approach and increases the duration of exposure to IFN\textalpha\textsubscript{-}2b. Recombinant adenosvirus (rAd)–IFN\textalpha\textsubscript{-}2b is a replication-deficient adenosvirus-based gene transfer vector that encodes the human IFN\textalpha\textsubscript{-}2b gene.\textsuperscript{22-24} Syn3, a polyamide surfactant, is incorporated into the drug formulation (rAd–IFN\textalpha\textsubscript{-}2b/Syn3; Instiladrin, FKD Therapies Oy, Kuopio, Finland)\textsuperscript{25} to enhance adenoviral transduction of the bladder lining. Dramatic enrichment of rAd–IFN\textalpha\textsubscript{-}2b gene transfer and expression has been shown with Syn3 in both normal urothelium and human urothelial carcinoma that grows in mice.\textsuperscript{22-25} rAd–IFN\textalpha\textsubscript{-}2b gene therapy mimics the physiologic events associated with viral infection, which results in local rather than systemic IFN\textalpha\textsubscript{-}2b production and subsequent tumor regression.\textsuperscript{22}

A phase I dose-ascending study of rAd–IFN\textalpha\textsubscript{-}2b/Syn3 was performed for patients with BCG-refractory and relapsing NMIBC.\textsuperscript{26} Dose-dependent adenoaviral gene transfer and urine concentrations of IFN\textalpha\textsubscript{-}2b were confirmed. Of 14 patients treated with dose levels of rAd–IFN\textalpha\textsubscript{-}2b/Syn3 that resulted in measurable urine IFN\textalpha\textsubscript{-}2b, six (43%) were free from recurrence at 3 months and had no dose-limiting toxicity, and two patients remained disease free at 29 and 39 months.\textsuperscript{26} These provocative findings, predominantly at the two highest doses, prompted this phase II study, designed to evaluate the efficacy and safety of intravesical rAd–IFN\textalpha\textsubscript{-}2b/Syn3 for patients with HG NMIBC refractory to, or with relapse after, BCG.

### Methods

#### Study Design

This randomized, open-label, parallel-arm study was conducted across 13 centers in the United States between November 5, 2012, and April 8, 2015. The protocol, administrative oversight, and accrual timelines were designed and conducted by the Society of Urologic Oncology Clinical Trials Consortium. The study protocol and informed consent form were reviewed and approved by the respective responsible site institutional review boards and biosafety committees.

#### Patients

The trial was designed to enroll 40 patients unable or unwilling to undergo radical cystectomy, and there were two dosage groups of 20 patients each. Eligible patients were 18 years or older and had HG BCG-refractory or relapsed NMIBC, including papillary NMIBC alone (Ta or T1), carcinoma in situ (CIS) alone, or a combination of CIS and papillary disease. BCG-refractory disease was defined as the inability to achieve a disease-free state at 6 months after adequate induction BCG therapy with either maintenance or reinduction at 3 months. Adequate induction was defined as a minimum of five of six treatments, and adequate maintenance was defined as a minimum of two of three treatments. BCG relapse was defined as recurrence within 1 year after a complete response to adequate BCG treatment (at least five and two instillations). Patients were required to have undergone visually complete resection of papillary lesions by transurethral resection of bladder tumors. Patients could not have received intravesical therapy within 3 months before beginning study treatment, with the exception of cytotoxic agents when administered as a single instillation immediately after a transurethral resection. All participants who entered the study provided written or oral informed consent.

#### Random Assignment and Masking

Patients were assigned by computer-generated random assignment, with a constrained 1:1 sequence, to receive either low-dose (\(1 \times 10^{11}\) viral particles [vp]/ml) or high-dose (\(3 \times 10^{11}\) vp/ml) rAd–IFN\textalpha\textsubscript{-}2b/Syn3. These doses were the most promising observed in the phase I study. The total doses administered were 7.5 \(\times 10^{12}\) vp in the low-dose group and 2.25 \(\times 10^{13}\) vp in the high-dose group. Treatment allocation was performed centrally with a block size of two for all patients who had successfully completed screening, with the constraint that the first four patients at each site were balanced between cohorts.

#### Procedures

rAd–IFN\textalpha\textsubscript{-}2b/Syn3 in 75 ml was administered intravesically through a urethral catheter, with a planned retention time of 1 hour; an anticholinergic treatment was allowed to relieve urinary urgency and permit adequate retention. Patients without recurrence of HG disease at months 3, 6, and 9, as evaluated by cytology, cystoscopy, and biopsy (if clinically indicated) were then retreated at months 4, 7, and 10. At 12 months, a final efficacy evaluation was performed. This evaluation included a protocol-mandated biopsy from the site of the index tumor and at least five random biopsies, including the bladder dome, trigone, right and left lateral wall, posterior wall, and prostatic urethra in men with positive cytology or prior disease in this region.

During the study, patients were contacted weekly by phone for the first month after each treatment on days 7, 14 (of months 7 and 10 only), 21, and 28 (± 1 day) to provide information about adverse events (AEs) and concomitant medication use. Assessments for treatment failure were made between 14 and 7 days before retreatment. Patients who were withdrawn from treatment before study completion underwent a safety assessment at least 30 days after last administration of the study drug. All patients are being monitored in a 3-year-long-term follow-up period to (1) determine recurrence of HG disease in those patients with a complete response and (2) to assess the long-term impact of treatment with rAd–IFN\textalpha\textsubscript{-}2b/Syn3.

#### End Points

The primary end point was freedom from HG disease recurrence at 12 months, defined by a negative for cause or end of study biopsy. Secondary end points included response to treatment, defined as no evidence of recurrence of HG disease at 3, 6, and 9 months; incidence and time to cystectomy; and concentration of IFN\textalpha\textsubscript{-}2b in the urine. Safety assessments included physical examination, monitoring of vital signs, ECG, and standard clinical chemistry; hematology, and urinalysis assessments (performed by local laboratories). Safety end points include type, incidence, relatedness, and severity of AEs and severe (≥ grade 3) AEs (SAEs), as assessed by National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.03).

#### Statistical Analyses

We determined that a cohort of 20 patients would be sufficient to give an 80% probability of rejecting a HG recurrence-free survival (RFS) rate of 10% with an exact 5% one-sided test when the true HG RFS rate was 35%. The operating characteristics for this Fleming design were calculated exactly with the binomial distribution described by Altman.\textsuperscript{27} The hypothesis—that the response rate was equal to or less than the reference rate—was rejected if five or more of the 20 patients achieved HG RFS at 12 months. The proportion of patients who achieved HG RFS at 3, 6, 9, and 12 months was reported for each dose group, together with an exact 90% CI for the proportion. The time to HG recurrence or death was summarized with the Kaplan-Meier method. Analyses were performed with SAS (version 9 or later; SAS Institute, Cary, NC). Both the safety and efficacy (modified

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intention-to-treat) analysis sets included all patients who received at least one dose of rAd–IFNα/Syn3. A data monitoring committee oversaw the study according to the data monitoring plan.

Analytical Assays and Sample Testing
All analytical assays were developed and validated. Samples were tested according to good laboratory practices methods at Covance Laboratories Ltd (Harrogate, United Kingdom). Description of the assays and the results of sample testing are presented in the Appendix (online only).

RESULTS
Patient disposition is shown in Figure 1. Baseline characteristics are listed in Table 1.

Primary End Point: HG RFS
The 12-month HG RFS rate was comparable between the two dose groups, with 33.3% of patients (7 of 21; 90% CI, 16.8 to 53.6) in the low-dose group and 36.8% (7 of 19; CI, 18.8 to 58.2) in the high-dose group alive and free of HG disease at 12 months. Overall, 35.0% of patients (14 of 40; 90% CI, 22.6% to 49.2%) remained free of HG recurrence at 12 months after the initiation of rAd–IFNα/Syn3 treatment (Table 2). Off-schedule disease assessments did not affect findings (Appendix, online only). The median time to HG recurrence or death was 6.5 months (90% CI, 3.52 to 12.78 months); the median time to HG recurrence was 3.52 months (90% CI, 3.02 to 12.78 months) for the low-dose group and was 11.73 months (90% CI, 5.88 months to not evaluable) for the high-dose group.

When patient subgroups and secondary end points were considered in exploratory analyses, the 12-month HG RFS rates were broadly similar for men and women, for younger and older patients, for refractory or relapsed NMIBC, for CIS only or papillary tumors and CIS, and for patients with Ta and T1 disease only (Table 2). Interestingly, of the 14 patients who were recurrence free at 12 months, 10 (71%) of the 14 had an antiadenovirus antibody response (defined as four times the predose titer), compared with 11 (24%) of 25 who experienced recurrence.

Significant levels of urine IFNα-2b were measurable in all patients in month 1 at days 2, 4, and 12 (Table 3). Of those patients who received a second dose, measurable IFNα-2b urine concentrations were noted in month 4 on days 2 and 4 after drug administration. Urine IFNα-2b concentrations did not appear to correlate with dose or clinical response.

In long-term follow-up, seven patients (18%) who withdrew from the study because of HG disease recurrence within the 12-month study period died at a median of 16 months (range, 2 to 26 months) after the withdrawal date. There is no indication that these deaths were treatment related. The cause of death was unknown in four patients, whereas two died as a result of progressive bladder cancer and one died as a result of liver failure unrelated to treatment 17 months after withdrawal from the study. The four patients for whom the cause of death is unknown were being observed locally after they completed their end-of-study evaluation. Fourteen patients (35%) who experienced an HG recurrence within the first year underwent a radical cystectomy at a median of 9 months (range, 4 to 28 months) from day 1 of month 1.

Patients are being monitored for 3 years to collect long-term follow-up data. Of the 14 patients who remained disease free at 12 months, additional follow-up data are being collected for 11; 3 withdrew from the study. Nine of these 11 patients are alive, and eight remained disease-free during a period of 15 to more than 36 months (Table 4). Two patients experienced HG recurrence at 21 and 28 months, respectively, from the start of treatment. One of these patients who experienced progression to muscle invasion underwent a radical cystectomy 31 months after the initiation of treatment and later died at 41 months. The other, who experienced recurrence at
21 months, remained alive and free from distant recurrence at 36 months. One patient free from bladder recurrence at 12 months died as a result of an upper tract tumor at 17 months.

Safety End Points

Overall, 39 patients (97.5%) experienced AEs during the study; 20 patients (95%) were in the low-dose arm, and 19 patients (100%) were in the high-dose arm (Data Supplement). In 34 of these patients (85%), at least one AE was considered to be drug related; in 18 (87.5%) of 21 patients in the low-dose arm and in 16 (84.2%) of 19 patients in the high-dose arm. The most frequently reported drug-related AEs were micturition urgency in 16 patients (84.2%) of 19 patients in the high-dose arm. The most frequently related AEs: in 18 (87.5%) of 21 patients in the low-dose arm and in 16 (85%) of 20 patients in the high-dose arm re...
disease that improves disease-specific patient outcomes and avoids cystectomy.

We recognize that this study is limited by its relatively small sample size and the lack of a comparative treatment arm. However, the trial was designed to determine optimal dosing and to provide preliminary efficacy to develop a definitive single-arm registration study. Few agents have actually gone beyond phase I and II development, so it is readily apparent that the traditional pathway for drug registration does not work for NMIBC. This concern was addressed through deliberations among the Society of Urologic Oncology, American Urological Association, and FDA, with the consensus that a single-arm trial with a mixed population of papillary disease and CIS was appropriate for the BCG-unresponsive population, given that a minimal threshold of patients who had some disease and CIS was met.17

Although the clinical impact of rAd–IFNα is encouraging, the mechanisms that mediate its antitumor activity remain undefined. In preclinical studies, IFNα and rAd–IFNβ inhibited angiogenesis, 28,29 and IFNα directly induced apoptosis in human bladder cancer cells by inducing autocrine tumor necrosis factor–related apoptosis-inducing ligand production. 30 Furthermore, rAd–IFNα overcame resistance to the IFNα protein in vitro and in animal models. It is now well established that IFNα controls dendritic cell maturation and antigen presentation and promotes tumor recognition by T cells and natural killer cells, and that these effects likely play more important roles in tumor growth inhibition than the direct effects of IFNα on tumor cells.31–33 Like IFN gamma, IFNα induces programmed death ligand 1 expression,34 which may limit tumor immune recognition and almost certainly inhibits T-cell activation; this may

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### Table 2. Incidence of HG RFS at 3, 6, 9, and 12 Months

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall (N = 40)</th>
<th>1 × 10^11 vp/mL (n = 21)</th>
<th>3 × 10^11 vp/mL (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFS at secondary end point analysis time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>10 (47.6) 28.6 to 67.2</td>
<td>13 (68.4) 47.0 to 65.3</td>
<td>23 (57.5) 43.3 to 70.6</td>
</tr>
<tr>
<td>6 months</td>
<td>8 (38.1) 20.6 to 58.3</td>
<td>9 (47.4) 27.4 to 68.0</td>
<td>17 (42.5) 29.2 to 56.7</td>
</tr>
<tr>
<td>9 months</td>
<td>8 (38.1) 20.6 to 58.3</td>
<td>9 (47.4) 27.4 to 68.0</td>
<td>17 (42.5) 29.2 to 56.7</td>
</tr>
<tr>
<td>12 months</td>
<td>7 (33.3) 16.8 to 53.8</td>
<td>7 (36.8) 18.8 to 58.2</td>
<td>14 (35.0) 22.6 to 49.2</td>
</tr>
</tbody>
</table>

HG recurrence-free subgroup at 12 months

- Refractory NMIBC (n = 31)
- Relapsed NMIBC (n = 19)
- CIS only (n = 21)
- Papillary tumor (n = 9)
- Ta + T1 disease only (n = 10)

Serum antiadenoviral antibody

- Positive (n = 22)
- Negative (n = 17)

Abbreviations: CIS, carcinoma in situ; HG, high-grade; NMIBC, non–muscle-invasive bladder cancer; rAd–IFNα/Syn3, recombinant adenovirus interferon alpha protein/Syn3 (a nonreplicating recombinant adenovirus gene transfer vector for patients with high-grade bacillus Calmette-Guerin–refractory or relapsed NMIBC); RFS, relapse-free survival; Ta: papillary urothelial carcinoma confined to the mucosa; T1: micro invasive urothelial carcinoma invasive into lamina propria but not muscularis propria; vp, viral particles.

*CI is for the proportion of patients with HG RFS; 90% CIs are based on the exact binomial method.

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### Table 3. Urinary IFNα-2b Concentrations After Treatment With rAd–IFNα/Syn3

<table>
<thead>
<tr>
<th>Visit</th>
<th>No. (%) of Patients</th>
<th>90% CI (%)</th>
<th>No. (%) of Patients</th>
<th>90% CI (%)</th>
<th>No. (%) of Patients</th>
<th>90% CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MID1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MID2</td>
<td>40</td>
<td>100</td>
<td>247-68,255</td>
<td>118-91,441</td>
<td>34-922</td>
<td>34-922</td>
</tr>
<tr>
<td>MID4</td>
<td>34</td>
<td>85</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MID12</td>
<td>7</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M4D1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M4D2</td>
<td>17</td>
<td>74</td>
<td>54-11,587</td>
<td>34-1,329</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M4D4</td>
<td>8</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M4D12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: IFNα-2b, interferon alpha-2b protein; MID1, month 1 day 1; MID2, month 1 day 2; MID4, month 1 day 4; MID12, month 1 day 12; M4D1, month 4 day 1; M4D2, month 4 day 2; M4D4, month 4 day 4; M4D12, month 4 day 12; rAd–IFNα/Syn3, recombinant adenovirus interferon alpha protein/Syn3 (a nonreplicating recombinant adenovirus gene transfer vector for patients with high-grade bacillus Calmette-Guerin–refractory or relapsed non–muscle-invasive bladder cancer).

*Urinary IFNα-2b concentrations were measured by ELISA. Concentrations were measured over 2 dosing cycles and the data are presented as both the number of patients with measurable IFNα-2b concentrations in each dosing cycle and the range of measurable protein concentrations in IU/mL.
explain the resistance to rAd–IFNα by some of the bladder cancers treated in this study. Combination therapy with IFNα and an anti–programmed death 1 inhibitor was more efficacious in preclinical studies than either agent alone at inhibition of melanoma tumor growth, and combination trials in NMIBC are under consideration.34 Finally, studies have demonstrated that local delivery of IFNα is better than systemic delivery to enhance tumor immune recognition, and viral transduction itself provides an important signal for kickstarting the immune system. Thus, in addition to serving as a bioreactor for sustained IFNα production and viral activation of intracellular pattern receptors. Thus, there are multiple reasons to explain the enhanced efficacy of rAd–IFNα compared with IFNα-2b in the treatment of refractory NMIBC.35

In summary, rAd–IFNα/Syn3 was well tolerated and demonstrated promising efficacy for patients with HG NMIBC after BCG therapy. A phase III trial of high-dose rAd–IFNα/Syn3, which provided longer median HG RFS and equivalent biosafety, is ongoing.

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### AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at jco.org.

### AUTHOR CONTRIBUTIONS

Conception and design: Seth P. Lerner, Alan Boyd, F. Peter Treasure, Gillian Gregory, David G. Sawutz, Seppo Yla-Herttuala, Nigel R. Parker
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

<table>
<thead>
<tr>
<th>Stage at Entry</th>
<th>Dose Group</th>
<th>Duration of Bladder HG RFS Since Day 1 (months)</th>
<th>Time of Last follow-up from Day 1 (months)</th>
<th>Status at Last Follow-Up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta/CIS</td>
<td>High</td>
<td>21</td>
<td>47</td>
<td>Recurrence of HGD</td>
</tr>
<tr>
<td>Ta</td>
<td>Low</td>
<td>28</td>
<td>41</td>
<td>Recurrence at 28 months</td>
</tr>
<tr>
<td>CIS</td>
<td>Low</td>
<td>15</td>
<td>15</td>
<td>Died at 41 months</td>
</tr>
<tr>
<td>Ta/CIS</td>
<td>Low</td>
<td>30</td>
<td>36</td>
<td>Recurrence of HGD</td>
</tr>
<tr>
<td>Ta</td>
<td>High</td>
<td>16</td>
<td>16</td>
<td>CR</td>
</tr>
<tr>
<td>T1/CIS</td>
<td>Low</td>
<td>35</td>
<td>37</td>
<td>CR</td>
</tr>
<tr>
<td>T1</td>
<td>Low</td>
<td>30</td>
<td>50</td>
<td>CR</td>
</tr>
<tr>
<td>CIS</td>
<td>High</td>
<td>38</td>
<td>39</td>
<td>CR</td>
</tr>
<tr>
<td>CIS</td>
<td>High</td>
<td>34</td>
<td>37</td>
<td>CR</td>
</tr>
<tr>
<td>CIS</td>
<td>Low</td>
<td>27</td>
<td>27</td>
<td>CR</td>
</tr>
<tr>
<td>T1/CIS</td>
<td>Low</td>
<td>17</td>
<td>17</td>
<td>Died of upper tract recurrence</td>
</tr>
<tr>
<td>Ta</td>
<td>High</td>
<td>13</td>
<td>13</td>
<td>CR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Withdraw</td>
</tr>
</tbody>
</table>

NOTE. Duration of HG RFS represent the number of months from day 1 that a complete response within the bladder has been documented based on yearly reports. Three patients withdrew from the study shortly after the 1-month end-of-study evaluation. Two patients had recurrence of HGD at 21 and 28 months from day 1. One of these patients underwent a cystectomy but later died. One patient died of an upper tract tumor without a bladder recurrence.

Abbreviations: CIS, carcinoma in situ; CR, complete response; HG, high-grade; HGD, high-grade disease; rAd–IFNα/Syn3, recombinant adenosine interferon alfa protein/Syn3 (a nonreplicating recombinant adenosine gene transfer vector for patients with HG bacillus Calmette-Guerin–refractory or relapsed non–muscle-invasive bladder cancer); RFS, relapse-free survival; Ta, papillary urothelial carcinoma confined to the mucosa; T1, micro-invasive urothelial carcinoma invasive into lamina propria but not muscularis propria.
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Appendix

Supplemental Methods

Role of the funding source. FKD Therapies Oy (Kuopio, Finland) provided funding to the investigators for study design, conduct, treatment administration, and data collection. The study database was held by the funder. All authors had unrestricted access to the raw and final study data and were responsible for data interpretation, the preparation of the report, and the decision to submit for publication.

Recombinant Adenovirus Interferon Alfa Protein/Syn3 production. First-generation replication-deficient serotype 5 adenovirus vector, which expressed human interferon alfa-2b (IFNα-2b) cDNA under a cytomegalovirus promoter, was produced under good manufacturing practice conditions in 293 cells, as previously described,1 with slight modifications of the process. It was tested to be free of endotoxin, microbiologic contaminants, and other impurities. The structure of the vector was verified by sequencing. Production of recombinant IFNα-2b was verified from each production lot with immunologic methods. The excipient Syn3 is a polyamide surfactant that enhances adenoviral gene transfer to the bladder epithelium.2,3

Analytical Assays

Sample collections and assay methods. Whole blood and urine samples were collected on days 1 (predose), 2, 4, and 12 of months 1 and 4 for measurement of recombinant adenovirus IFNα-2b (rAd–IFNα-2b) DNA and IFNα-2b concentrations (urine only). Serum samples for IFNα-2b protein concentration measurements also were collected on days 1 (predose), 2, 4, and 12 of months 1 and 4. Serum samples for antibody assays were collected before dosing on day 1 of months 1, 4, 7, and 10, as well as at the month-13/withdrawal visit.

Urine samples for rAd–IFNα-2b DNA, IFNα-2b, and exploratory assays were collected into a sterile container and stabilized with the addition of buffer that contained 10% bovine serum albumin and 50 mM of HEPES (pH, 7.4). Two mL of buffer was added to each 20-mL sample of urine as soon as possible after collection of the urine sample. After addition of the stabilization buffer, aliquots were transferred into 2-mL cryotubes by using sterile pipette tips and were put on ice. Whole-blood samples for determination of rAd–IFN DNA by polymerase chain reaction (PCR) were collected into EDTA-containing tubes. Blood samples were collected at the required time points, were divided into sterile polypropylene cryotubes with sterile pipette tips, and were frozen at −70°C until shipment for analysis. Whole-blood samples for serum IFNα-2b measurements and for determination of anti-adenoviral and anti–IFNα-2b antibodies were drawn at the required time points. The samples were drawn into red top Vacutainer (Becton, Dickinson, and Co., Franklin Lakes, NJ) tubes and allowed to clot at room temperature for 30 minutes. The samples were then centrifuged at 4°C, × 1,500 g, for 15 minutes, and the serum was separated into cryovials. All samples for all assays were frozen at −70°C within 5 hours of collection and were stored for shipment and analysis.

IFNα-2b protein concentration assay. Measurement of IFNα-2b concentrations in urine and serum samples was done by ELISA with a MesoScale discovery platform (Meso Scale Diagnostics, Bethesda, MD). Samples were incubated with a master mix to allow the IFNα-2b to bind to biotinylated-anti–IFNα antibodies and sulfo-tagged (sTag)–anti-IFNα antibodies to form an antibody-bridge complex. After incubation, samples were added to the streptavidin-coated plate. The biotinylated–anti-IFNα antibodies bound to the streptavidin-coated plate, which allowed any unbound material to be washed away. Read buffer that contained tripropyamine was added. The sTag associated with anti-IFNα antibodies produced a chemiluminescent signal when an electrical voltage was applied. The concentration of IFNα-2b in samples was then back-calculated from a calibration curve. The method had a lower level of quantification of 31 IU/mL and an upper level of quantification of 2,000 IU/mL.

Analytical assays for rAd–IFNα DNA in blood and urine. To assess systemic exposure and urinary concentrations of rAd–IFNα vector DNA, a sensitive and specific quantitative PCR (qPCR) assay for the vector DNA was developed and validated. In both assay matrices, amplification was detected in all replicates of the standard curve (1 × 10^9 viral particles [vp]/225 μL to 1 × 10^3 vp/225 μL for all valid runs), and the correlation coefficient of the dilutions (R^2) was greater than or equal to 0.98 for all qPCRs performed. An assessment of the specificity of the qPCR assay was made with human and Escherichia coli DNA. No cross reactivity with either matrix was observed when 1-, 0.5-, and 0.1-μg templates were present in the qPCR assay. To determine if either human or E. coli DNA could interfere with the accuracy of the qPCR assay, a spike of 2 × 10^3 vp/2 μL of rAd–IFNα DNA (derived from the appropriate matrix matched standard) was spiked into a background of each concentration of genomic DNA.

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**Supplemental Results**

*Sensitivity analysis of primary end point.* To assess the impact of off-schedule disease assessments on the primary efficacy end point, a sensitivity analysis was conducted in which 12 months was defined according to the assessment date as opposed to the nominal month-13 assessment. Results for the sensitivity analysis were identical to the primary efficacy end point: 14 of 40 patients (35%) overall showed high-grade recurrence free survival at 12 months and experienced comparable incidences for the dose groups (low-dose: n = 7 of 21 [33%]; high-dose: n = 7 of 19 [37%]).

**IFNα-2b serum concentrations.** Serum IFNα-2b concentrations were low. At day 2 of month 1, 31 of the 40 patients had concentrations less than 31 IU/mL (limit of assay quantification). Six patients had concentrations greater than 31 IU/mL but less than 50 IU/mL, and two patients had concentrations greater than 50 IU/mL but less than 160 IU/mL.

**Blood and Urine rAd–IFNα DNA measurements.** Median blood and urine concentrations of rAd DNA were measured with a qPCR assay that had a level of detection of $1 \times 10^3$ vp/225 μL. Importantly, no measurable rAd–IFNα DNA was detected in blood after the initial dosing. Of the 23 patients who received a second dose at month 4, only one patient (4.3%), randomly assigned to the $3 \times 10^{11}$ vp/mL dose group, had a positive test result for a low level of virus detected at day 2 of month 1 ($7.7 \times 10^3$ vp/225 μL), which was not measurable by day 4 of month 1.

As expected, all 40 patients had significant copies of rAd DNA in their urine at day 2 of month 1; the median value was $1.13 \times 10^6$ vp/225 μL. Thirty-nine patients had measurable concentrations of rAd DNA copies at day 4 of month 1. However, these were approximately three orders of magnitude lower; the median value was $8.08 \times 10^5$ vp/225 μL. Thirty-three patients (85%) had measurable concentrations at day 12 of month 1, and the median value was $2.3 \times 10^5$ vp/225 μL. In the 23 patients who received a second dose of rAd–IFN, 22 had measurable concentrations of rAd DNA at day 2 of month 4 and a median value of $5.13 \times 10^5$ vp/225 μL, and 20 patients had measurable concentrations of approximately eight times the level of detection at day 4 of month 4 and a median value of $8.45 \times 10^5$ vp/225 μL. By day 12 of month 4, only six patients (29%) had measurable copies of rAd–IFNα DNA in the urine. Results for the two dose cohorts were comparable.

**Anti-IFNα antibody and antiadenvirus antibody concentrations.** Anti–IFNα-2b antibody concentrations in serum were measured in serum from each patient. With the sole exception of one patient who had a weak 1:20 titer at day 12 of month 1, no other patient at any time point had measurable anti–IFNα-2b antibodies. Antiadenvirus type 5 antibody concentrations were measured in serum from each patient with a quasi-quantitative assay (see Covance Laboratories, Harrogate, UK for details).

Antibody data were collected at days 1 and 12 of month 1, day 1 of month 7, day 1 of month 10, and at the month-13 withdrawal assessment. The data demonstrated that 22 patients (55.0%) had a significant antiadenvirus antibody response (defined as four times the predose titer). Of the 14 patients who experienced a complete response, 10 (71%) had a significant antiadenvirus antibody response, and four (29%) did not demonstrate a significant response. These data suggest that a significant antiadenvirus vector antibody response does not appear to correlate with lack of efficacy. A definitive antibody titer for any of the positive patients was not determined.

**Safety**

A summary of all treatment-emergent adverse events is provided in the Data Supplement.