Phase II Clinical Trial of Intralesional Administration of the Oncolytic Adenovirus ONYX-015 in Patients with Hepatobiliary Tumors with Correlative p53 Studies

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ABSTRACT

Purpose: ONYX-015 is a genetically modified adenovirus with a deletion of the E1B early gene and is therefore designed to replicate preferentially in p53-mutated cells. A Phase II trial of intralesional ONYX-015 was conducted in patients with hepatobiliary tumors to determine the safety and efficacy of such a treatment.

Experimental Design: All patients had biopsy-proven, measurable tumors of the liver, gall bladder, or bile ducts that were beyond the scope of surgical resection. Patients received intralesional injections of ONYX-015 at either $6 \times 10^8$ or $1 \times 10^{10}$ plaque-forming units/lesion up to a total dose of $3 \times 10^{10}$ plaque-forming units, and i.p. injections were allowed in patients with malignant ascites. The status of p53 was assessed by immunohistochemistry or Affymetrix GeneChip microarray analysis. Studies were conducted for viral shedding and for the presence of antiadenoviral antibodies before and after the injection of ONYX-015. Patients were assessed for response and toxicity.

Results: Twenty patients were enrolled, and 19 patients were eligible. Half of the patients had primary bile duct carcinomas. Serious toxicities (> grade 2) were uncommon and included hepatic toxicity (three patients), anemia (one patient), infection (one patient), and cardiac toxicity (one patient, atrial fibrillation). Sixteen patients were evaluable for response. Among these evaluable patients, 1 of 16 (6.3%) had a partial response, 1 of 16 (6.3%) had prolonged disease stabilization (49 weeks), and 8 of 16 (50%) had a >50% reduction in tumor markers. Of the 19 eligible patients, 18 (94.7%) had specimens available for p53 analysis. Fifteen of these 18 patients (83.3%) had evidence of p53 mutation by one or both methods, although the methods correlated poorly. Viral shedding was confined to bile (two of two patients) and ascites (four of four patients). Pretreatment adenoviral antibodies were present in 14 of 14 patients and increased by 33.2% after ONYX-015 treatment.

Conclusions: Intralesional treatment with ONYX-015 in patients with hepatobiliary tumors is safe and well tolerated, and some patients had evidence of an anticancer effect. The high incidence of p53 mutations in these tumors makes this a logical population in which to test this therapy but precludes definitive evaluation about the necessity of a p53 mutation for ONYX-015 clinical activity.

INTRODUCTION

ONYX-015 (d1520, CI-1042) is a type 2, type 5 chimeric Ad that has been genetically modified by disruption of the coding sequence of the M, 55,000 E1B protein and by insertion of point mutations that generate stop codons in the early coding sequences to prevent the expression of N-terminal protein fragments that might have biological activity (1). These modifications were designed to allow ONYX-015 to replicate preferentially in tumors with defects in the p53 pathway, which, in addition to mutations in p53 itself, could also include loss of p14ARF function or overexpression of MDM2; the selectivity of this approach derives from the fact that nearly all nonneoplastic tissues have wild-type p53 (2). ONYX-015 has demonstrated antineoplastic effects in vivo against a wide range of human tumor cells, including numerous carcinoma lines with either mutant or normal p53 gene sequences (3, 4). In a Phase I clinical trial of intralesional injection in patients with head and neck cancer, dose-limiting toxicity was not reached at $1 \times 10^{11}$ pfu, and ONYX-015 was able to be administered safely, with a predominant toxicity of fevers in 21% of patients (5). In Phase II trials, administered as a single agent (6) or in combination with chemotherapy (7), ONYX-015 has demonstrated clinical activity. There-

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1 The abbreviations used are: Ad, adenovirus; pfu, plaque-forming unit(s); IHC, immunohistochemistry; CT, computed tomography; ECOG, Eastern Cooperative Oncology Group; FNA, fine-needle aspiration; CR, complete response; PR, partial response; PD, progressive disease; SD, stable disease; CPE, cytopathic effect; PBST, phosphate-buffered saline with 0.1% Tween-20.
fore, it has been proposed that this agent be studied in tumors refractory to conventional therapeutic agents (8).

Cancers of the liver, bile ducts, and gall bladder are an important clinical problem worldwide and will account for more than 17,000 deaths in the United States in 2002 (9). Whereas each has a distinct natural history, etiology, and clinical course, tumors of the liver, bile ducts, and gall bladder share several common features. They are embryologically related (10) and locally aggressive tumors (11), and once beyond the scope of surgical resection, they are generally refractory to nearly all commonly used chemotherapeutic agents (12, 13). Furthermore, these tumors have a high rate of \( p53 \) mutation (14–17), suggesting that they may be attractive candidates for \( p53 \)-targeted therapy such as ONYX-015.

To investigate this question, we initiated a Phase II trial of ONYX-015 in patients with hepatobiliary tumors to assess the antitumor efficacy and toxicity of this agent. Because these tumors are generally locally invasive, intralesional therapy was administered. The status of \( p53 \) was evaluated by complementary methods, IHC and Affymetrix GeneChip analysis, which uses microarray technology to detect mutations in exons 2–11 of the gene.

Current concerns regarding therapy that uses a replicating Ad include the risk of infection to patient contacts, especially those who are immunosuppressed, and the potential futility of using an Ad in patients who already have antiviral antibodies. To address the first question, we collected body fluids and measured viral shedding at various time points after viral injection. To address the second question, antiviral antibody levels were measured before treatment and again at 1–3 weeks after injection. Our results demonstrate that intralesional injection of ONYX-015 can be administered safely in patients with hepatobiliary tumors with evidence of antineoplastic effects despite the presence of antiviral antibodies and that the risk of horizontal transmission is probably low. The prevalence of \( p53 \) mutations in these tumors was higher than expected using the combination of IHC and Affymetrix analysis.

PATIENTS AND METHODS

Administrative. This was a single-institution, prospective Phase II trial. The protocol was approved by the Investigational Drug Branch of the Cancer Therapy Evaluation Program (National Cancer Institute, NIH), by the Protocol Review Committee of the Albert Einstein Cancer Center, and by the Institutional Review Board and Biosafety Committees of the Montefiore Medical Center.

Eligibility. Patients were required to have an advanced or metastatic carcinoma of the hepatobiliary system (including hepatocellular carcinoma, cholangiocarcinoma, carcinoma of the gallbladder, or carcinoma of the ampulla of Vater) that was beyond the scope of surgical resection. All patients had measurable disease, accessible by either CT-guided percutaneous needle aspiration or endoscopy, ECOG performance status of 0 or 1, and adequate organ function, defined as a leukocyte count \( \geq 3.5 \times 10^9/\text{mm}^3 \), platelets \( \geq 100,000/\text{mm}^3 \), serum creatinine \( \leq 2.0 \text{ mg/dl} \), and aspartate aminotransferase and alanine aminotransferase \( \leq 4 \times \) the upper limits of normal. Patients with an elevated total bilirubin \( \geq 3 \times \) the upper limit of normal due to biliary obstruction by tumor were eligible for this trial. Patients were allowed to have received \( \leq 2 \) prior chemotherapeutic regimens but must have had no treatment for at least 4 weeks before study entry. Patients who were carriers of hepatitis B were excluded from protocol entry to eliminate the theoretical possibility that inactivation of \( p53 \) by the hepatitis B X antigen might lead to replication of ONYX-015 in normal liver parenchyma of hepatitis B carriers (18). Other exclusion criteria included replacement of >50% of liver by tumor, coagulopathy that could not be easily corrected to a prothrombin time \( <15 \text{ s} \), prior malignancy within 5 years (other than resected basal cell carcinoma of the skin), family history of malignancy suggestive of Li-Fraumeni Syndrome, brain metastases, presence of active infection or other uncontrolled comorbid condition, pregnancy or lactation, requirement for immunosuppressive or antiviral (including antiretroviral) medication, and prior treatment with adenoviral vectors. All patients signed an informed consent form approved by the Montefiore Medical Center Institutional Review Board.

Treatment. Patients were admitted to the hospital 1 day before ONYX-015 administration. To prevent abscess formation in lesions injected with ONYX-015 that might undergo rapid necrosis, the first eight patients treated received prophylactic antibiotic coverage with oral erythromycin (1 g every 6 h) and kanamycin (1 g every 6 h), beginning on the day before ONYX-015 administration and continuing for 24 h after treatment. In addition, patients received i.v. ticarcillin/clavulanate (3.1 g every 4 h) and gentamycin (80 mg every 8 h). Patients allergic to penicillin received vancomycin (1 g every 12 h), aztreonam (2 g every 8 h), and metronidazole (500 mg every 6 h). Antibiotics began on the day before ONYX-015 administration and continued for 1 week after treatment. The protocol was subsequently amended to eliminate prophylactic antibiotics, and the remaining patients received antibiotics only in the case of presumed infection.

ONYX-015 was administered intratumorally in the radiology suite under computed tomographic guidance (Fig. 1, A and B). The dose of drug was fixed and was not adjusted for body surface area, but it was adjusted for the projected volume of the lesions. The first two patients were initially treated with a total dose of \( 6 \times 10^9 \) pfu. After these patients demonstrated no significant toxicity, the dose of ONYX-015 was escalated to \( 1 \times 10^{10} \) pfu/lesion, with a maximum dose of \( 3 \times 10^{10} \) pfu. The volume of injection was one-third of the total tumor volume, delivered into one to three areas of the lesion. Specifically, lesions <2 cm received one injection, lesions of 2–4 cm received two injections, and lesions >4 cm received three injections. Patients with malignant ascites could also receive i.p. ONYX-015 at a dose of \( 1 \times 10^{10} \) pfu.

Immediately before ONYX-015 treatment, a FNA was performed at the injection site for histological confirmation of tumor at the site (Fig. 1D), as well as for \( p53 \) analysis by both IHC (Fig. 1E) and \( p53 \) probe array. Samples for \( p53 \) Affymetrix analysis were snap frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). A maximum of three lesions (including ascites) were treated at one time. Patients with more than three injectable lesions were eligible to return 2 weeks after the original treatment to receive treatment of up to three additional lesions.
Patients who tolerated treatment and did not develop PD were eligible for retreatment at 3-week intervals.

The first eight patients treated were observed in the hospital on i.v. antibiotics and under contact and droplet isolation protocols for 1 week after receiving ONYX-015. In the absence of both massive hepatic necrosis and evidence of viral shedding, the protocol was amended to allow stable patients to be discharged on the day after ONYX-015 administration.

Samples of urine, sputum, and, when possible, bile and ascites were obtained daily for 1 week after the first ONYX-015 treatment to assess the presence of viral shedding. Serum was also obtained from a subset of these patients before treatment and at 1 and 3 weeks after the first treatment for evaluation of Ad-specific antibodies.

**Response Evaluation.** Patients were evaluated for response every 6 weeks, by CT scan and tumor markers (CEA, CA19-9, CA125, and, in hepatoma patients, AFP). Radiographic response was defined as follows: CR was defined as disappearance of all radiographic evidence of tumor, normalization of all tumor markers, and stabilization or improvement in performance status, persisting for at least 4 weeks. PR was defined as an at least 50% reduction in the sum of the products of the perpendiculars of all measurable lesions without the appearance of new lesions, stabilization or improvement in all tumor markers, and stabilization or improvement in performance status, persisting for at least 4 weeks. PD was defined as an at least 25% increase in the sum of the products of the perpendiculars of all measurable lesions. Appearance of a new lesion was not considered PD if all injected lesions were stable or responding and the patient was clinically stable. Patients not meeting criteria for CR, PR, or PD were classified as SD.

Because of the difficulty of measuring drug effect on liver lesions and specifically because many of these lesions were highly scirrhous with only minimal nests of tumor cells (Fig. 1C; Ref. 11), biochemical assessment of response was also measured. Tumor marker response was defined as a 50% reduction in at least one serum tumor marker that was elevated pretherapy, associated with SD radiographically and stable or improved performance status.

**Analysis of p53 Status.** Because ONYX-015 is postulated to replicate only in tumors in which p53 is mutated or in which the p53 pathway is deregulated, p53 status was assessed by both IHC and Affymetrix GeneChip analysis. Lesions to be injected with ONYX-15 were individually sampled by CT-guided FNA biopsy. A pathologist was present in the radiology suite at the time of FNA to ensure that material procured from radiologically localized lesions was representative of the carcinoma. FNA material from each pass was immediately smeared onto several charged slides, although the number of slides prepared was dependent on the volume of material aspirated. At least one slide prepared from each site was air dried and immediately stained with Diff-Quick stain for microscopic adequacy assessment. If Diff-Quick-stained material was deemed nondiagnostic or insufficient, additional passes from the lesion were requested of the radiologist. All remaining slides were fixed in 95% ethanol and subsequently designated for either p53 IHC or routine Papanicolaou staining. Slides for IHC were fixed for 20–30 min, air dried, and processed for staining on the same or the subsequent day. IHC staining with p53 antibody (diluted 1:50; DAKO) was performed on the DAKO Autostainer Universal Staining System and developed using the DAKO EnVision mouse peroxidase kit with DAKO DAB chromogen. Brown nuclear staining of any intensity within tumor cells was interpreted as evidence of p53 immunoreactivity. There was generally insufficient tissue for cellblock preparation, and the limited volume of material obtained also precluded the use of smears as negative controls.

**Analysis of p53 by Affymetrix GeneChip.** Qiagen DNA Mini kits (Qiagen, Valencia, CA) were used to extract genomic DNA from the same CT-guided FNA used for IHC. These samples were placed on ice at the time of acquisition. The genomic DNA was amplified with PCR using the GeneChip p53
primer set (Affymetrix, Santa Clara, CA) and AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) according to the Affymetrix instructions for p53 target preparation. The coding regions of the human p53 gene were amplified as 10 separate amplicons in a single multiplex reaction. The DNA amplicons were then fragmented using the GeneChip Fragmentation Reagent (Affymetrix) according to the manufacturer’s instructions. Fragmented DNA amplicons were labeled at their 3’ ends with a fluorescein-dideoxyxynucleotide (fluorescein-ddCTP) and the BioArray terminal labeling kit for DNA probe array assays (Enzo Diagnostics, Farmingdale, NY) according to manufacturer’s instructions. Labeled fragments were then placed in hybridization buffer [6× saline-sodium phosphate-EDTA, 0.05% Triton X-100 (Sigma, St. Louis, MO), 2 mg/ml acetylated BSA (Life Technologies, Inc., Grand Island, NY), and 2× control oligonucleotide F1 (Affymetrix)]. GeneChip p53 probe arrays (Affymetrix; Ref. 19) were hybridized to labeled fragments and washed on GeneChip Fluidics Station 400 according to the manufacturer’s instructions. The probe scans were prepared (GeneArray Scanner 2508; Affymetrix) and analyzed using the Microarray Suite software version 5.0 (Affymetrix).

Analysis of Viral Shedding. Because of concerns about transmission of ONYX-015 via secretion into body fluids, viral shedding was measured in urine, ascites, bile, and peripheral blood. Initially, attempts were made to collect sputum; however, this was unsuccessful. Two assays were used. In the CPE assay, HEK293 human embryonic kidney cells were grown to 90% confluence in a 12.5-cm3 plug seal flask. Two hundred HEK293 human embryonic kidney cells were grown to 90% confluence in a 12.5-cm3 plug seal flask. Two hundred

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Statistical Analysis. Sample size was calculated based on Simon’s “minimax” design (20). Standard ECOG response criteria were used to measure response to treatment (21). In the first stage, 13 patients were accrued. If no patients respond, the study is closed, and a response rate of 20% is ruled out with 95% confidence. If at least one response is observed among the first 13 patients, 14 additional patients are accrued. If the true response rate is at least 20%, this would be detected with 95% confidence at a power of 80%.

RESULTS

Patient Demographics. Based on the planned statistical design of the trial, there was one responder among the first 14 patients, and the plan was to continue the trial to 36 patients; however, the trial was terminated administratively because of drug shortage. As shown in Table 1, 20 patients were enrolled. Half of the patients had bile duct tumors (cholangiocarcinoma or carcinoma of the ampulla of Vater). Most of the patients were previously untreated, and all were ambulatory. For one patient, who never received treatment, the diagnosis could not be hist-
Table 1 Demographics

<table>
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<tr>
<td>Enrolled patients</td>
<td>20</td>
</tr>
<tr>
<td>Eligible patients</td>
<td>19</td>
</tr>
<tr>
<td>Evaluable patients</td>
<td>16</td>
</tr>
<tr>
<td>Male:female</td>
<td>11:9</td>
</tr>
<tr>
<td>Age (yrs)</td>
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<tr>
<td>Median</td>
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</tr>
<tr>
<td>Range</td>
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<tr>
<td>ECOG performance status</td>
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</tr>
<tr>
<td>Site of primary lesion</td>
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</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>9</td>
</tr>
<tr>
<td>Gallbladder carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Carcinoma of the ampulla</td>
<td>1</td>
</tr>
<tr>
<td>of Vater</td>
<td></td>
</tr>
<tr>
<td>Prior chemotherapy</td>
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<td></td>
<td>12:6:2</td>
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</table>

No tissue confirmation in one patient.

Table 2 Toxocities (n = 19)

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<tr>
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<th>National Cancer Institute common toxicity criteria grade</th>
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<tr>
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<td>0  1  2  3  4</td>
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<tr>
<td>Leukopenia</td>
<td>17 1 1 0 0</td>
</tr>
<tr>
<td>Anemia</td>
<td>17 0 1 1 0</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>16 3 0 0 0</td>
</tr>
<tr>
<td>Fever</td>
<td>6  4 9 0 0</td>
</tr>
<tr>
<td>Myalgia</td>
<td>10 6 3 0 0</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>12 7 0 0 0</td>
</tr>
<tr>
<td>Infection</td>
<td>18 0 0 0 1</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>16 1 2 0 0</td>
</tr>
<tr>
<td>Hepatic</td>
<td>14 1 1 2 1</td>
</tr>
<tr>
<td>Cardiac</td>
<td>18 0 0 1 0</td>
</tr>
<tr>
<td>Arthritis</td>
<td>18 0 1 0 0</td>
</tr>
<tr>
<td>Hypertension</td>
<td>18 0 1 0 0</td>
</tr>
<tr>
<td>Hypotension</td>
<td>18 0 1 0 0</td>
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</tbody>
</table>

Table 3 Response to treatment (n = 19)

<table>
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<tr>
<th></th>
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<tbody>
<tr>
<td>CR</td>
<td>0</td>
</tr>
<tr>
<td>PR</td>
<td>1</td>
</tr>
<tr>
<td>Reduction in tumor markers by &gt;50%</td>
<td>8</td>
</tr>
<tr>
<td>SD</td>
<td>12</td>
</tr>
<tr>
<td>PD</td>
<td>3</td>
</tr>
<tr>
<td>Not evaluable</td>
<td>3</td>
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</tbody>
</table>

disease progression rather than viral-induced liver failure. Likewise, the single grade 4 infection was related to tumor progression resulting in sepsis. Postinjection fever, chills, and myalgias were frequent but mild and self-limited. Hematological toxicity also was mild. There were no complications resulting from the injection, including bleeding or infection.

Response to Treatment. Among the 19 eligible patients, 16 were evaluable for response to therapy. Three patients died before completing their first response assessment of causes felt to be unrelated to study treatment. The first, a 74-year-old man with metastatic cholangiocarcinoma and portal hypertension, died of a variceal bleed 10 days after receiving ONYX-015 treatment. The second, a 43-year-old woman with cholangiocarcinoma, died of a cerebrovascular accident 22 days after receiving ONYX-015. The third patient, a 43-year-old woman with cholangiocarcinoma, developed sudden onset of hypothermia, hypotension, and metabolic acidosis 14 days after ONYX-015 treatment and died of presumed sepsis 24 h later. No causative organism was identified.

Among the 16 patients evaluable for response, there was one (6%) PR in an injected lesion lasting 13.5 weeks (Table 3). In addition, 8 of 16 (50%) evaluable patients had declines of at least 50% in at least one serum tumor marker, associated with SD radiographically, and stable or improved performance status. These “tumor marker responses” lasted for a median of 11.5 weeks (range, 6.5–20.5 weeks). One additional patient, who had recurrent cholangiocarcinoma, exhibited a prolonged period of SD (radiographically, clinically, and via tumor markers) for 49 weeks.

Analysis of p53 Status. Among the 19 evaluable patients, p53 status was analyzed by two methods. A positive signal on IHC indicates the presence of a mutation in the genome, which stabilizes the protein and increases the normally short half-life. This methodology was used and was informative in 17 of the 19 patients, of whom 9 (52.9%) exhibited a positive signal for p53 (Table 4). Affymetrix GeneChip uses microarray technology to analyze the entire coding region of p53 (exons 2–11). The microarray assay identifies nonsense mutations and single-base deletions and can identify mutant p53 in a background of wild-type p53. Eighteen of 19 patients had DNA samples that were informative by GeneChip analysis. Of these, 11 of 18 (61.1%) demonstrated mutations in p53 (Table 4). When patient samples were analyzed for mutation in p53 by either IHC or Affymetrix, 15 of 18 (83.3%) samples were positive. Nevertheless, there was poor correlation between the techniques: only 7 of 17 (41.1%) samples analyzable and informative for both techniques were concordant (5 samples were positive by both techniques, and 2 samples were negative by both techniques).

To confirm the safety and tolerability of ONYX-015 intralésional therapy, a planned dose escalation was incorporated into the study design. The first two patients received 6 × 10⁹ pfu/lesion, and the same patients were subsequently escalated to a dose of 1 × 10¹⁰ pfu/lesion with a maximum dose of 3 × 10¹⁰ pfu. The remaining patients received 1 × 10¹⁰ pfu/lesion with a planned maximum dose of 3 × 10¹⁰ pfu, which was delivered to all subsequent patients.

Toxicities. As shown in Table 2, among the 19 eligible patients, therapy was well tolerated. The one episode of cardiac toxicity was atrial fibrillation, which was asymptomatic and required medical therapy only. In retrospect, this patient’s atrial fibrillation had likely predated her treatment with ONYX-015. Hepatic toxicity generally resolved spontaneously when related to therapy or, alternatively, was secondary to disease progression. No patients had sequelea from altered liver functions, and the single incidence of grade 4 hepatic toxicity was related to
Correlation of p53 Status with Response. As shown in Table 4, it is difficult to correlate p53 status with response because 83.3% of patients had evidence of p53 abnormalities by at least one technique. However, it is interesting to note that the only radiographic response to this agent was seen in patient 11, who had only a borderline positive signal for p53 overexpression (~1%) by IHC and no evidence of mutation by Affymetrix analysis. Patient 2, who had prolonged stabilization of disease, had no evidence for mutation in p53. Among the eight tumor marker responders, seven (patients 1, 7, 8, 9, 13, 17, and 20) had specimens informative for p53 status by at least one method (six of eight specimens were informative for p53 status by IHC, and seven of eight were informative for p53 status by Affymetrix). Five of seven (71.4%) responders had a p53 mutation by at least one method (three of six had a p53 mutation by IHC, and six of seven had a p53 mutation by Affymetrix). Among the six evaluable patients who were neither tumor marker responders nor had a PR or prolonged stabilization (patients 3, 5, 6, 10, 18, and 19), six of six had a p53 mutation by one or both methods (three of six had a p53 mutation by IHC, and four of six had a p53 mutation by Affymetrix).

To account for the absence of concordance between IHC and Affymetrix analysis, specific mutations were analyzed (Table 5). In patient 1, p53 was overexpressed, and a Q167E missense mutation was observed in a codon within the zinc-binding domain by microarray analysis. In patient 2, no aberrancies in p53 were observed by either technique. In patients 3, 5, and 15, no mutations were observed by microarray analysis, but overexpression of p53 was observed by IHC. In patients 4 and 6, null mutations in regions before the p53 tetramerization domain, which were therefore likely inactivating mutations, were detected by microarray analysis in the absence of overexpression by IHC.

Patient 7 had three missense mutations, two within the S2-S2’ β strand, with no overexpression of p53 by IHC. In patients 8 and 18, both a polymorphism and a missense mutation outside of a conserved region of the p53 genome were observed; in patient 8, this was associated with overexpression of p53 by IHC, but in patient 18, overexpression of p53 by IHC was not observed. Patient 9 had four missense mutations by microarray analysis in the absence of overexpression of p53 by IHC. Two mutations, S241C and R273P, were in bases that directly contact DNA, and one mutation, E286Q, was in the H2-helix region of the molecule. Patient 10, who was also without overexpression of p53, had a polymorphism and an E286Q mutation also in the H2-helix region. Patient 11 had no mutations detectable by microarray analysis and had borderline p53 overexpression (0.7 ± 1.1%) by IHC.

Patient 14 had two missense mutations, one in a conserved region IV and an R273P mutation in the p53 DNA binding region. Patient 17 had two missense mutations, one of which was also in region IV. Patient 19 had a sole missense mutation in R273P. Both patients also had overexpression of p53 by IHC.

Patients with either a missense or null mutation in p53 by microarray analysis or overexpression of p53 by IHC were considered to have a mutation. Only two patients (patients 2 and 20) demonstrated wild-type p53 by both techniques. Patient 11, who had a negative microarray analysis and overexpression of p53 in <1% of cells analyzed, was considered borderline positive.

Antiadenoviral Antibody Studies. The presence of Ad-specific antibody levels was evaluated by ELISA before ONYX-015 treatment in the first 14 patients enrolled on trial. All patients demonstrated Ad-specific antibodies. Eight of these patients were also evaluated for Ad-specific antibodies at 1–3 weeks after their first treatment. Antibody levels increased by a
mean of 33.2 ± 10.3% after treatment, consistent with other trials (22). No relationship between tumor marker decline and Ad-specific antibodies (either pretreatment level or posttreatment increase) was observed.

Cell-mediated immune response was not formally measured; however, in one patient with a biliary tumor who died, the autopsy specimen revealed dense lymphoid aggregates in the area of lymphatic drainage for the injection site (Fig. 2). No Ad was detectable by immunohistochemical staining in either the primary tumor or the lymphatic drainage in the liver.

Analysis of Viral Shedding in Body Fluids. Posttreatment samples of serum, urine, and, where possible, ascites and biliary fluid were obtained from the first 14 patients enrolled on trial and assayed for viral shedding by CPE assay and PCR. Originally, we had planned to assay patient sputum; however, this strategy had to be abandoned due to the negligible amount of sputum produced by patients in the postinjection period.

Fifty-five samples of patient urine were obtained from 12 patients at various time points between 1 and 14 days postinjection of ONYX-015 and analyzed for viral shedding by CPE assay. No specimen showed any evidence of viral shedding. PCR was not performed on urine samples due to technical difficulties with the procedure. Two patients had biliary stents available for analysis of shedding into bile. Both had evidence of viral shedding into bile by PCR analysis at 1–5 days after injection of ONYX-015. Of the four patients with ascites, two had positive CPE assays at 1–5 days, and all four had evidence of virus by PCR analysis at 1–9 days postinjection.

DISCUSSION

The most important finding of this study was that ONYX-015 can safely be administered intralesionally in patients with hepatobiliary tumors and that some patients had evidence of therapeutic effect as measured by radiological response, prolonged stabilization of disease, or a ≥50% decrease in levels of tumor markers. Given the highly scirrhous histology of these tumors (Fig. 1), biochemical evidence of response by measurement of declines in tumor markers may be a reasonable alternative to radiographic measurement of response, although by no means are the two equivalent. The lack of serious toxicities in this population and the findings of a favorable clinical outcome in about half of the patients suggest that intralesional therapy...
with ONYX-015, despite the cumbersome nature of the CT-guided injections, is nevertheless worth exploring further either as a single-agent therapy or in combination with cytotoxic agents for which there is evidence for synergy (3, 7, 22–25).

Other than surgery or liver transplantation, there are few effective therapeutic options for patients with hepatobiliary tumors. One small trial of ONYX-015 (dl1520) injection in patients with hepatocellular carcinoma suggested some modest level of clinical benefit with minimal toxicities (27). Because most of the studies with ONYX-015 have been performed in patients with head and neck, ovarian, or pancreatic cancer, our trial was novel and suggests a clinical benefit in hepatobiliary tumors. Additional studies should be considered in this group of patients.

Despite the presumed selectivity of the virus for p53-mutated cells (essentially excluding normal tissues), the widespread prevalence of antiadenoviral antibodies in the normal population, and the benign nature of adenoviral infection, horizontal transmission of virus is a concern with ONYX-015 therapy. The viral shedding studies, although limited, are therefore encouraging. Whereas we were unable to obtain adequate sputum for viral studies, the absence of viral shedding in the urine suggests effective systemic clearance of the virus. One concern, however, is the presence of virus in the bile; additional studies with stool samples may be warranted. Shedding into ascites was also of interest, not in terms of horizontal transmission, but in terms of potential for antineoplastic effects against i.p. tumors.

The nearly universal presence of antiadenoviral antibodies in our population demonstrates that the therapeutic effect of ONYX-015 is not necessarily compromised by intact humoral immunity, although in the absence of a comparable group of patients without preexisting antiadenoviral antibodies, it is not possible to definitively exclude an antibody-mediated decrement in the clinical activity of the virus. The efficacy of our approach may relate to the intralesional route of injection, which may compartmentalize the virus at least temporarily from the immune response. Alternatively, the ability of ONYX-015 to enter and replicate in tumor cells without immediately lysing them may create a sanctuary for viral replication within the p53-mutated or possibly even the p53 wild-type tumor cells. Thus, the bolus injection of concentrated virus directly into tumors may allow binding and entry of virus into tumor cells before the immune response can be mobilized, and virus may subsequently replicate intracellularly, spared from humoral or cell-mediated immune response. Data generated using in situ RT PCR analysis of ONYX-015 injected into two abdominal wall implants support this hypothesis (26).

One controversial question regarding the utility of ONYX-015 is the degree of selectivity for p53-mutated cells. There is clear evidence that ONYX-015 can replicate in cells with wild-type p53 (3, 25, 28, 29). Others have argued, however, that the p53 pathways in these cells may be disrupted because of decreased levels of p14ARF or elevated levels of MDM2, which
could functionally inactivate p53 (30–32). There is also evidence that ONXY-015 can infect cells regardless of p53 status but can replicate more efficiently in p53-mutated cells (33).

To investigate the status of p53 in the patients enrolled in this trial, two methods of mutation detection were used, microarray analysis using the Affymetrix GeneChip, which has provided good agreement with direct manual sequencing (34), and IHC. A principal finding of our study was the absence of correlation between the microarray analysis and the IHC analysis. Six patients with mutations detected by microarray analysis had no overexpression of p53 by IHC. Three had null mutations located before the tetramerization domain and would not be expected to produce functional proteins. Because IHC detects mutant proteins based on epitope recognition, truncated proteins may be missed with this method. The other three cases are more difficult to explain. Two patients had missense mutations in codons for amino acids in direct contact with the DNA binding domain or in the H2-helix regions, which are likely inactivating lesions. This is confirmed by clinical studies in which patients with missense mutations in the protein-DNA binding domain, H2-helix, or loop structures had a substantially worse prognosis than patients with wild-type p53 (35). The fifth patient had both a polymorphism and a missense mutation outside a conserved region and had no overexpression of p53 by IHC. These patients also tend to have a worse prognosis than patients with wild-type p53 but have an improved prognosis when compared with patients with mutations in the conserved regions (35). There were also three patients with overexpression of p53 ranging from 12.4–19.6% of analyzed cells, who did not have mutations detected by microarray analysis. Because the GeneChip algorithm is not optimized for detection of intragenic deletions or insertions, this is the likely explanation for this observation (36).

One interesting finding from this study is that 3 of 18 patients had identical g–c mutations in codon 273, substituting a proline for an arginine. This R273P mutation was of interest for several reasons: (a) mutations at codon 273 are the most common mutations in p53; (b) the arginine at 273 is one of the amino acids in direct contact with DNA in the DNA-protein binding region of p53; and (c) the fact that this mutation occurred in nearly 19% of patients, a rate that appeared to be higher than that which would have occurred by chance. Two databases of p53 mutations, one from the WHO, which listed 15,329 mutations (IARC TP53 mutation database, IARC, WHO, Lyon, France), and one from the Institut Curie, which listed 14,969 mutations (T. Soussi, C. Gallou, and C. Beroud, p53 database, Laboratoire de Genatoxologie des Tumeurs, Institut Curie, Paris, France) failed to correlate the R273P mutation with tumors of the liver, gall bladder, or bile duct, and several studies of patients with hepatobiliary tumors failed to demonstrate the presence of this mutation (37–39). This observation may indicate a novel p53 mutation but will require confirmation by direct sequencing.

In summary, our study showed that intralésional injection of ONXY-015 in patients with hepatobiliary tumors was safe and well tolerated. Over half of the patients had some evidence of clinical benefit, despite the universal presence of antadenoviral antibodies before treatment. Although limited, shedding studies showed no risk for horizontal transmission of virus, making this therapy applicable to the clinic. Finally, mutations in p53 were detected in >80% of patients who received treatment at least one of two methods, making any decision about the necessity of p53 mutation for viral replication moot. The absence of concordance between IHC and the Affymetrix GeneChip array analysis was a concern for future studies, and neither may provide the ultimate answer (40). Additional studies to explore the best way to analyze p53 are warranted.

REFERENCES

ONYX-015 Therapy for Hepatobiliary Tumors

Clinical Cancer Research

Phase II Clinical Trial of Intralesional Administration of the Oncolytic Adenovirus ONYX-015 in Patients with Hepatobiliary Tumors with Correlative p53 Studies

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