

Phase II Clinical Trial of Intralesional Administration of the Oncolytic Adenovirus ONYX-015 in Patients with Hepatobiliary Tumors with Correlative *p53* Studies¹

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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 6×10^9 or 1×10^{10} plaque-forming units/lesion up to a total dose of 3×10^{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 (dl1520, CI-1042) is a type 2, type 5 chimeric Ad³ that has been genetically modified by disruption of the coding sequence of the M_r 55,000 E1B protein and by insertion of point mutations that generate stop codons in the early coding sequences to prevent the expression of NH₂-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 p14^{ARF} 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 vitro* 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 1×10^{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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³The 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 antiadenoviral 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, antiadenoviral 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 antiadenoviral 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 $\geq 3.5/\text{mm}^3$, platelets $\geq 100,000/\text{mm}^3$, serum creatinine ≤ 2.0 mg/dl, and aspartate aminotransferase and alanine aminotransferase $\leq 4\times$ the upper limits of normal. Patients with an elevated total bilirubin $> 3\times$ the upper limit of normal due to

biliary obstruction by tumor were eligible for this trial. Patients were allowed to have received ≤ 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 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 6×10^9 pfu. After these patients demonstrated no significant toxicity, the dose of ONYX-015 was escalated to 1×10^{10} pfu/lesion, with a maximum dose of 3×10^{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 1×10^{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°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.

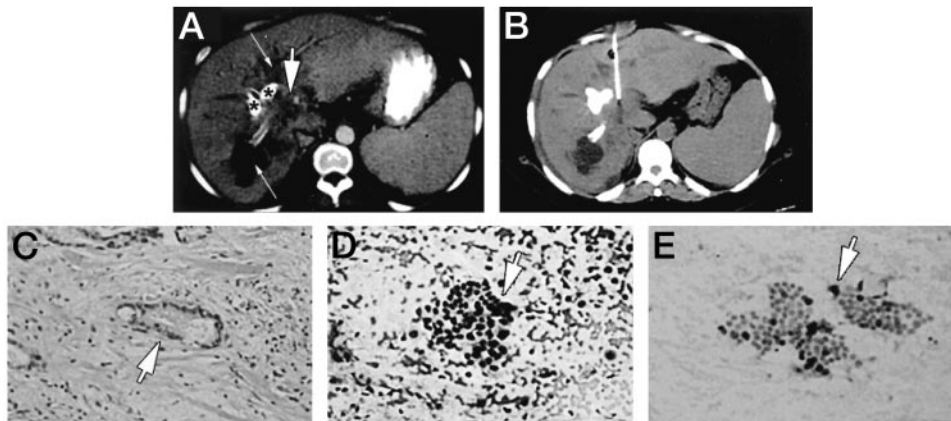


Fig. 1 Administration of ONYX-015 to a patient with a cholangiocarcinoma. **A**, contrast-enhanced CT scan shows a tumor in the porta hepatis (*thick arrow*) extending into the right and left intrahepatic bile ducts. There are multiple biliary stents (*asterisks*) with adjacent bilomas (*thin arrows*). **B**, unenhanced CT scan 1 week later shows a 20-gauge spinal needle used for injection of ONYX-015 into the tumor. **C**, histological specimen demonstrates nests of glandular tumor cells in scirrhous background (*arrow*). **D**, cytologic specimen from the same site as shown in **B** demonstrates the presence of adenocarcinoma (*arrow*). **E**, immunohistochemical staining of specimen from the same site as shown in **B** for p53 demonstrates dark cells that overexpress p53 (*arrow*).

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% reduc-

tion 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 fluoresceinated dideoxynucleotide (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 nM 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 arrays were scanned (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 90–100% confluence in a 12.5-cm³ plug seal flask. Two hundred and fifty μ l of patient ascites, bile, urine, or serum were diluted in 200 μ l of DMEM. When necessary, pH was adjusted to 7.2–7.4 with 50 μ l of sodium bicarbonate [7.5% (w/v)]. Media covering the cells were removed, and 500 μ l of the diluted samples were added. The samples were incubated at 37°C with 5% CO₂ for 1 h, with gentle rocking every 20 min. After incubation, 10 volumes (5 ml) of DMEM containing 2.5% fetal bovine serum, 4500 mg/liter glutamine, 100 mIU/ml penicillin, and 100 μ g/ml streptomycin were added. Cells were inspected daily for evidence of CPE for up to 14 days. If CPE was apparent, cells were harvested and stored at –80°C. Frozen cells were thawed at 37°C, and cellular debris was removed by centrifugation at 453 × *g* for 15 min. Supernatant was filtered through a 0.22 μ m nitrocellulose filter, and 500 μ l of the filtered supernatant were then used to reinfect confluent HEK293 cells. After the second infection, 10 volumes (5 ml) of 2.5% fetal bovine serum in DMEM with glutamine, penicillin, streptomycin, and 2.5 μ g/ml Fungizone were added to each of the flasks. Where CPE was evident in the second infection, cells were harvested and stored at –80°C until DNA purification. Infection assays were scored positive for ONYX-015 only after verification by PCR. Contents of flasks in which CPE was witnessed after the second infection assay (above) were thawed and centrifuged to remove cellular debris. Supernatant (200 μ l) was used with the Qiagen DNA Blood Mini Kit, according to the manufacturer's recommended protocol. DNA was eluted in 35 μ l of nuclease-free water and stored at –80°C until PCR was performed. Every effort was made to prevent cross-contamination between samples at all times. For extraction from serum, ascites, and biliary drainage, 200 μ l of sample were used di-

rectly with the Qiagen kit according to the manufacturer's recommended protocol, with elution in 35 μ l of nuclease-free water.

The second assay used nested PCR to detect virus. PCR of purified viral DNA was performed with primers specific for the ONYX-015 virus. Both primer pairs are specific for a region of salmon sperm DNA inserted into the virus during its generation. This region is present on the plasmid dl309, which was used as a positive control for the PCR. The following primers were used to amplify a 585-bp region of the salmon sperm DNA: Int309S, ctgctccatgttgtgttgctaccat; and Int309AS, acctaccgggaagtcataaatgaac. PCR was carried out in 50 μ l containing 1× Taq buffer (Eppendorf), 1× Taq enhancer, 100 nM deoxynucleotide triphosphates, 100 nM each primer, 1.7 units of Taq polymerase (Eppendorf), and 10 μ l of purified viral DNA as template. The reactions were carried out at 95°C for 3 min, followed by 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 35 cycles, and concluded with a 10-min extension at 72°C. Two μ l of the initial PCR product were used as a template in a nested PCR reaction. Conditions for the nested PCR were the same as those for the first-round PCR. The following primer pair was used to amplify an internal 335-bp fragment of DNA: ne309S, cctttgcgttctggtaggccg; and ne309AS, ctgtgaagagcacagggcgcc. Gel electrophoresis was performed on the second-round PCR product. Thirty μ l of sample were run on a 0.8% agarose gel for detection of transcripts.

Detection of Ad-specific Antibodies by ELISA. ONYX-015 was diluted in PBS and stored overnight at 4°C at 1 × 10⁸ particles/well. Wells were washed five times with PBS + 0.1% Tween 20 and blocked for 1 h in PBST + 1% BSA at 37°C. Serial dilutions of serum samples were made in PBST + 0.1% BSA and added to wells in triplicate. After a 2-h incubation at 37°C, wells were washed three times, and antihuman horse radish peroxidase antibody (Promega) was added at a dilution of 1:10,000. After a 1-h incubation at 37°C, wells were washed three times, and O-phenylenediamine substrate was added. Fifteen min later, absorbance at 450 nm was read on a Bio-Rad Model 550 microplate reader.

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 his-

Table 1 Demographics

	<i>n</i>
Enrolled patients	20
Eligible patients	19 ^a
Evaluable patients	16
Male:female	11:9
Age (yrs)	
Median	60
Range	34–78
ECOG performance status 0:1	5:15
Site of primary lesion	
Cholangiocarcinoma	9
Gall bladder carcinoma	5
Hepatocellular carcinoma	5
Carcinoma of the ampulla of Vater	1
Prior chemotherapy 0:1:2	12:6:2

^a No tissue confirmation in one patient.

Table 2 Toxicities (*n* = 19)

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

tologically confirmed at the planned site of injection, and this patient was therefore ineligible. Among the remaining 19 patients, 49 cycles (mean, 3 cycles/patient; range, 0–6 cycles/patient) of therapy were administered. The median number of lesions treated was 3 (range, 0–6 lesions). In addition, four patients received i.p. therapy for malignant ascites.

To confirm the safety and tolerability of ONYX-015 intralesional therapy, a planned dose escalation was incorporated into the study design. The first two patients received 6×10^9 pfu/lesion, and the same patients were subsequently escalated to a dose of 1×10^{10} pfu/lesion with a maximum dose of 3×10^{10} pfu. The remaining patients received 1×10^{10} pfu/lesion with a planned maximum dose of 3×10^{10} 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 sequelae from altered liver functions, and the single incidence of grade 4 hepatic toxicity was related to

Table 3 Response to treatment (*n* = 19)

	<i>n</i>
CR	0
PR	1
Reduction in tumor markers by >50%	8
SD	12
PD	3
Not evaluable	3

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 was also 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 missense 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).

Table 4 Correlation of response with *p53* status

Patient no.	Eligible	Evaluable	Primary	OR ^a	TMR	IHC	Affy	Either	Concordance
1	Y	Y	Chol	N	Y	Y	Y	Y	Y
2	Y	Y	Chol	N	NE	N	N	N	Y
3	Y	Y	Chol	N	N	Y	N	Y	N
4	Y	N	Chol	NE	NE	N	Y	Y	N
5	Y	Y	Chol	N	N	Y	N	Y	N
6	Y	Y	Chol	N	N	N	Y	Y	N
7	Y	Y	Ampulla	N	Y	N	Y	Y	N
8	Y	Y	GB	N	Y	Y	Y	Y	Y
9	Y	Y	GB	N	Y	N	Y	Y	N
10	Y	Y	Hep	N	N	N	Y	Y	Y
11	Y	Y	Hep	Y	N	Y	N	Y	N
12	N	N	Hep	NE	NE	NE	NE	NE	NE
13	Y	Y	Hep	N	Y	NE	N	N	NE
14	Y	N	Chol	NE	NE	Y	Y	Y	Y
15	Y	N	Chol	NE	NE	Y	N	Y	N
16	Y	Y	Chol	N	Y	NE	NE	NE	NE
17	Y	Y	GB	N	Y	Y	Y	Y	Y
18	Y	Y	GB	N	N	N	Y	Y	N
19	Y	Y	GB	N	N	Y	Y	Y	Y
20	Y	Y	Hep	N	Y	N	N	N	N
Totals	19/20	16/19		1/16	8/15	9/17	11/18	15/18	7/17

OR, objective response; TMR, tumor marker response; Affy, Affymetrix GeneChip analysis; Either, either IHC or Affymetrix analysis positive for *p53* mutation; Concordance, both assays provide same results; Chol, cholangiocarcinoma; Ampulla, carcinoma of the ampulla of Vater; GB, gall bladder carcinoma; Hep, hepatocellular carcinoma; NE, not evaluable; Y, yes; N, no.

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 ($0.7 \pm 1.1\%$) by IHC.

Patient 14 had two missense mutations, one in 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

Table 5 Correlation of p53 mutation by IHC and Affymetrix analysis

Patient no.	Microarray exon	Mutation	Nucleotide position	AA change	IHC
1	Exon 5	ctg>ctg	517	L145R	13.7% (± 2.1)
	Exon 5	atg>ctg	573	M160L	
	Exon 5	cag>gag	604	Q167E	
	Intron 10	g>a	1368		
2	0	0			0
3	0	0			19.6% (± 9.4)
4	Exon 6	cga>tga	724	R196	0
5	0	0			12.4% (± 5.3)
6	Exon 8	cga>tga	1146	R306	0
	Intron 5	a>g	687		
7	Exon 5	caa>cac	488	Q136H	0
	Exon 5	gcc>ccc	492	A138P	
	Exon 5	ccc>ccg	542	P151	
8	Exon 6	cga>cgg	801	R213	11.4% (± 5.4)
	Exon 6	gtg>ctg	814	V217L	
9	Exon 7	tcc>tgc	906	S241C	0
	Exon 8	cgt>cct	1027	R273P	
	Exon 8	ggg>gcg	1053	G279A	
	Exon 8	gaa>caa	1080	E286Q	
10	Exon 4	ccg>ccc	166	P36	0
	Exon 8	gaa>caa	1080	E286Q	
	0	0			
11	0	0			0.7% (± 1.1)
12	NE ^a	NE	NE	NE	NE
13	0	0			NE
14	Exon 7	atg>atc	925	M246I	61%
	Exon 8	cgt>cct	1027	R273P	
	Intron 10	a>g	1367		
15	0	0			14.2% (± 8.9)
16	NE	NE	NE	NE	NE
17	Exon 7	atc>aac	956	I254N	95%
	Exon 8	ggg>gcg	1135	G302A	
18	Exon 6	cga>cgg	801	R213	0
	Exon 6	cat>gat	803	H214D	
19	Exon 8	cgt>cct	1027	R273P	Skin nodule: 84%, Periumbilical nodule: 58%
	Intron 8	g>t	1160		
20	Intron 6	g>a	838		NE

^a NE, not evaluable.

mean of $33.2 \pm 10.3\%$ 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 1–6 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 $\geq 50\%$ 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

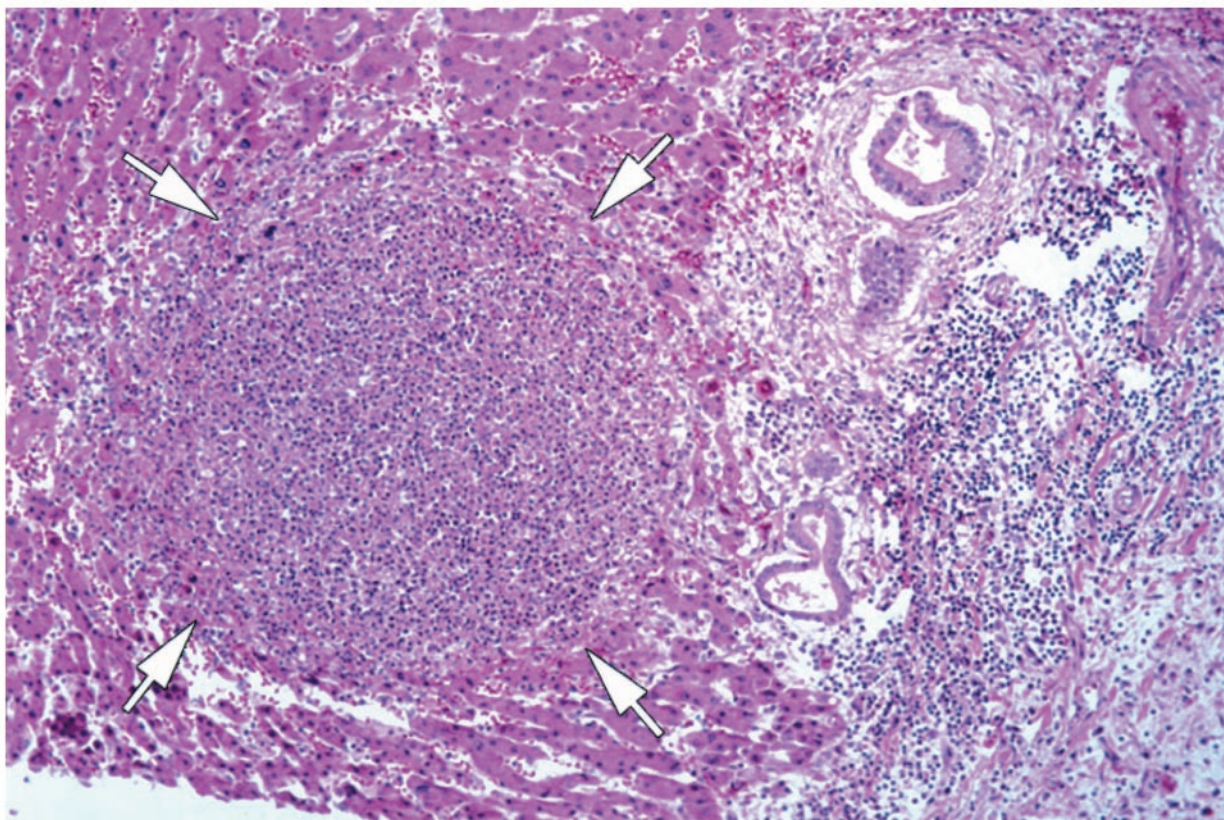


Fig. 2 Tissue section from autopsy specimen of a patient with a biliary tumor. The section shows normal liver with robust lymphocytic aggregation (arrows) at the site of the lymphatic drainage from the biliary tumor to the liver. No virus was present on routine immunohistochemical staining.

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 p14^{ARF} or elevated levels of MDM2, which

could functionally inactivate *p53* (30–32). There is also evidence that ONYX-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;⁵ IARC, WHO, Lyon, France), and one from the Institut Curie, which listed 14,969 mutations (T. Soussi, C. Gallou, and C. Beroud, *p53* database,⁶ Laboratoire de Genotoxicologie 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 intralesional injection of ONYX-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 antiadenoviral 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 by 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.

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⁵ www.iarc.fr/p53/index.html.

⁶ www.p53.curie.fr.

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