Clinical & Experimental Metastasis **18:** 379–384, 2001.

Antimetastatic efficacy of adjuvant gemcitabine in a pancreatic cancer orthotopic model

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Received 11 December 2000; accepted in revised form 30 January 2001

Key words: adjuvant therapy, gemcitabine, green fluorescent protein, metastasis, nude mice, pancreatectomy, pancreatic cancer, surgical orthotopic implantation

Abstract

Gemcitabine is a promising new agent that has been recently studied for palliation of advanced (stage IV) unresectable pancreatic cancer. We hypothesized that adjuvant gemcitabine would reduce recurrence and metastases following surgical resection of pancreatic cancer. To test this hypothesis, we evaluated gemcitabine on a green fluorescent protein (GFP) transductant of the human pancreatic cancer cell line BxPC-3 (BxPC-3-GFP) using surgical orthotopic implantation (SOI) in nude mice. GFP enabled high resolution fluorescent visualization of primary and metastatic growth. Five weeks after SOI, the mice were randomized into three groups: Group I received exploratory laparotomy only. Group II underwent surgical resection of the pancreatic tumor without further treatment. Group III underwent tumor resection followed by adjuvant treatment with gemcitabine, 100 mg/kg every three days for a total of four doses, starting two days after resection. The mice were sacrificed at thirteen weeks following implantation and the presence and location of recurrent tumor was recorded. Gemcitabine reduced the recurrence rate to 28.6% compared to 70.6% with resection only ($P = 0.02$) and reduced metastatic events 58% in the adjuvant group compared to resection only. This study, demonstrating that gemcitabine is effective as adjuvant chemotherapy post-pancreatectomy, suggests this new indication of the drug clinically.

Abbreviations: SOI – surgical orthotopic implantation; GFP – green fluorescent protein, 5-FU – 5-fluorouracil

Introduction

Pancreatic cancer is the fourth most common cause of cancer death in the United States, and the second most common cause of death from GI-related neoplasms [1]. The prognosis for this disease has remained dismal, even after curative attempts with surgical resection. Previously, 5-fluorouracil (5-FU) was the most widely used and studied chemotherapeutic agent for pancreatic cancer. Recently, gemcitabine has been shown to be more effective than 5-FU in alleviating disease-related symptoms and increasing survival in patients with advanced (stage IV) unresectable pancreatic cancer [2].

Surgical resection of pancreatic cancer remains the only curative option for this disease, but survival remains poor, even after pancreatectomy [3]. Most patients develop locoregional recurrences as well as hepatic and distant metastases after curative resection. To improve the low rate of cure of pancreatic cancer, several studies have been done on adjuvant and neoadjuvant chemotherapy and radiation. Most studies with adjuvant chemotherapy, however, have been performed using 5-fluorouracil. The Gastrointestinal Tumor Study Group demonstrated that adjuvant chemoradiation using 5-FU conferred a survival advantage (median survival of 20 months) versus 11 months with surgery alone [4]. Another study of adjuvant 5-FU post-pancreaticoduodenectomy showed an increased median survival of 19.5 months with adjuvant 5-FU compared to 13.5 months with no postoperative therapy [5]. However, other studies have shown little to no benefit of adjuvant chemotherapy with 5-FU alone, with a response rate of 10% or less.

Given the results of the recent trials showing enhanced efficacy of gemcitabine over 5-FU for palliative chemotherapy, we studied the use of adjuvant chemotherapy with gemcitabine in the setting of surgically resectable pancreatic cancer.

This study evaluated the efficacy of adjuvant gemcitabine using a clinically relevant mouse model developed from surgical orthotopic implantation (SOI) of human pancreatic adenocarcinoma [6–9]. This study utilized the BxPC-3 pancreatic tumor cell line transduced with green fluorescent

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protein (GFP) [10–17]. GFP expression allows for visualization and imaging of tumor growth and metastasis, an important factor for the understanding and treatment of the metastatic process.

Materials and methods

Pancreatic cancer cells

The BxPC-3 human pancreatic cancer cell line was obtained from the American Type Culture Collection (Rockville, Maryland). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B (Gibco-BRL, Life Technologies, Inc., Grand Island, New York). Cells were incubated at 37 °C in a 5% CO_2 incubator.

GFP transduction [18]

The RetroXpress vector pLEIN was purchased from Clontech Laboratories, Inc. (Palo Alto, California). The pLEIN vector expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contains an IRES site. pLEIN was produced in PT67 packaging cells. BxPC-3 cells were transduced with supernatants of the pLEIN-producing PT67 cells. Stable high-expression GFP transductants were selected in neomycin as previously described [18].

Surgical Orthotopic Implantation (SOI) [6–9]

Pancreatic tumors at the exponential growth phase, grown subcutaneously in nude mice, were resected aseptically and cut with scissors and minced into approximately 3 × 3 × 3 mm pieces as previously described [6–9]. Mice were anesthetized by isoflurane inhalation (Fort Dodge Animal Health, Fort Dodge, Iowa). The abdomen was sterilized with alcohol. A subcostal incision was then made through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed and three tumor pieces were transplanted on the middle of the pancreas with an 8-0 silk surgical suture (Ethicon Inc., Somerville, New Jersey). The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed with 6-0 silk suture. Animals were kept in a sterile environment. All procedures of the operation described above were performed with a ×7 microscope (Olympus).

At 5 weeks after orthotopic implantation, mice were randomized into 3 different groups of 18 mice each for treatment purposes (Figure 1). Group I underwent exploratory laparotomy only, without resection of the pancreatic tumor. Group II animals underwent resection of the pancreatic tumor via the same subcostal incision used for original implantation. Group III underwent resection as described above and postoperative adjuvant treatment with gemcitabine.

Resection of primary tumor

Tumor was removed by sharp dissection with scissors. Hemostasis was obtained with 8-0 silk sutures. The skin was closed with 6-0 silk sutures.

Fluorescence microscopy and image analysis (18)

A Leica stereo microscope MZ 12 equipped with a mercury bulb as a light source was used for the imaging experiments. Selective excitation of GFP was produced through a D425/60 band-pass filter and a 470 DCXR dichroic mirror. Fluorescence was emitted through a GG475 long-pass filter (Chroma Technology, Brattleboro, Vermont) and collected by a Hamamatsu Color Cooled CCD Video Camera HM C5810. High-resolution images were captured and processed with a Pro-Series Frame-Grabber and acquired by a Pentium-IV PC with Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, Maryland).

Gemcitabine treatment

Gemcitabine (Eli Lilly, Indianapolis, Indiana) was reconstituted in saline to a concentration of 5.2 mg/ml and administered intraperitoneally at a dose of 100 mg/kg/dose [2]. The first dose was given to Group III animals two days after resection. The dose was repeated every 3 days for a total of 4 doses. There was no apparent toxicity for this dose regimen as evidenced by mouse survival and body weight (data not shown).

Analysis of metastasis

At 13 weeks following SOI, mice were sacrificed and explored. Any recurrent tumor and all major organs were observed directly under fluorescence microscopy with images captured as described above. Weight of recurrent tumor and location of metastases were recorded for each mouse. The average number of metastatic events per mouse was calculated by dividing the total number of metastatic events per group by the number of mice per group.

Histological analysis

Recurrent tumor was removed, weighed, and saved for histologic analysis carried out with standard hematoxylin and eosin (H&E) staining.

Statistical analysis

The chi-squared test was used to measure differences between recurrence rates among the treatment groups (Microsoft Excel, Redmond, Washington). The t-test was used to measure differences in average tumor weight among the various groups (Microsoft Excel, Redmond, Washington). A $P \leq 0.05$ was considered to be statistically significant.

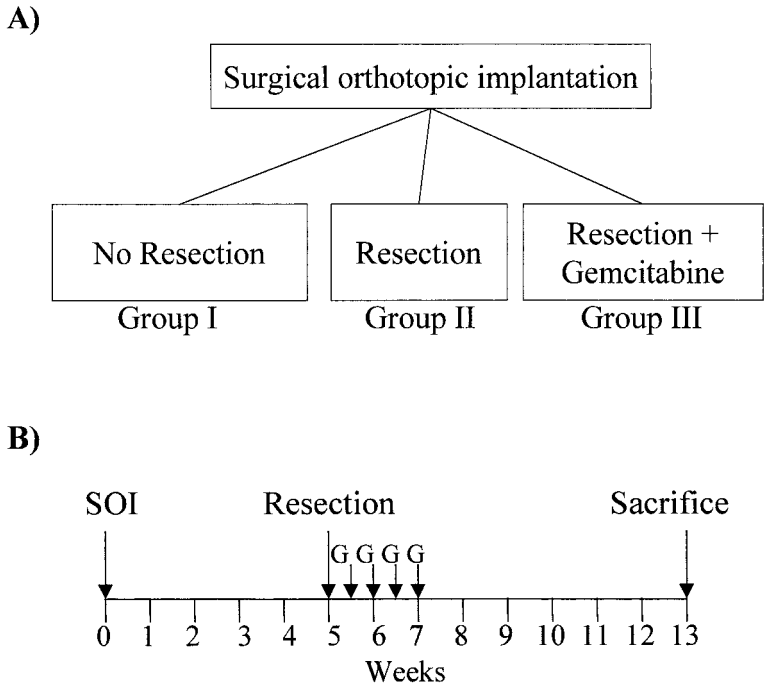

Figure 1. At 5 weeks following surgical orthotopic implantation, mice were randomly divided into three treatment groups. Group I underwent exploratory laparotomy without resection. Group II underwent surgical resection of the tumor. Group III underwent resection and received postoperative gemcitabine. B) Exploratory laparotomy (Group I) and resections (Groups II and III) were performed at 5 weeks after implantation. Group III received gemcitabine starting two days post-resection, with 100 mg/kg/dose q3d for a total of 4 doses. The mice were sacrificed at 13 weeks after implantation, and the presence of recurrent tumor and location of metastases were recorded.

Results and discussion

Tumor recurrence

The recurrence rate following resection alone (Group II) was 70.6%, whereas in the resection plus gemcitabine group (Group III), the recurrence rate was reduced to 28.6% (Figure 2). This 42% reduction in recurrence rate was statistically significant ($P = 0.02$, chi-squared test). The mean primary tumor weight was significantly higher for Group I (3492.3 mg) compared to Group II (1403.6 mg) ($P = 0.002$, t-test) and Group III (1829.2 mg) ($P = 0.02$, t-test). The difference in mean primary tumor weight between Group II and Group III was not statistically significant ($P = 0.36$, t-test).

Metastatic patterns

Figure 3 documents the location of metastases for each treatment group. In Group I, large tumors were noted in the pancreas as well as local and distant sites including spleen, retroperitoneum, portal nodes, diaphragm, small intestine, colon, liver, kidney, mediastinum, and lungs. In Group II, recurrences were also seen in the bed of the resected pancreas and in distant sites, including spleen, retroperitoneum, portal lymph nodes, diaphragm, small intestine, colon, mediastinum, and lungs. There was an average of 2.4 metastatic events per mouse. However, in Group III, significantly fewer metastases occurred, an average of 1 per mouse, ($P = 0.02$, chi-squared test), and there were no distant metastases to sites such as liver, kidney, mediastinum, and lungs. Recurrences in this group were limited to locoregional areas such as the spleen, retroperitoneum, portal lymph nodes, diaphragm, and bowel. The primary BxPC-3 tumor as well as the metastases were visualized using GFP (Figures 4A–C). Tumors were confirmed to be ductal adenocarcinoma by standard microscopy H&E stained sections (Figure 4D).

Bruns et al. recently described limited effects of gemcitabine in an orthotopic model of human pancreatic cancer in nude mice implanted with tumor cell suspensions [19, 20]. In these experiments, mice were treated with either gemcitabine or gemcitabine with an EGF-receptor inhibitor [19, 20]. In the present study, with the use of SOI to enhance metastasis and the addition of GFP as an aid to visualize metastasis of pancreatic cancer, we have made a significantly advanced metastatic model. For example, in the present study, metastases were visualized in the spleen, retroperitoneum, portal lymph nodes, diaphragm, small intestine, colon, liver, kidney, mediastinum, and lungs as opposed to only the liver and lymph nodes in the Bruns et al. studies [19, 20].

The present study has demonstrated adjuvant therapy with gemcitabine, in contrast to standard therapy with resection alone, reduced both the incidence and the rate of metastatic spread. Gemcitabine reduced the number of mice with recurrent tumor by 42% and reduced the metastatic rate by 58% per mouse compared to resection alone. Since surgical resection in humans still carries a high risk of recurrence, adding gemcitabine should allow for a higher rate of cure. The metastases in the mice treated with gemcitabine tended to occur locally in the retroperitoneum, spleen and diaphragm as opposed to extensive distant metastases in the untreated mice. This indicates that adjuvant gemcitabine

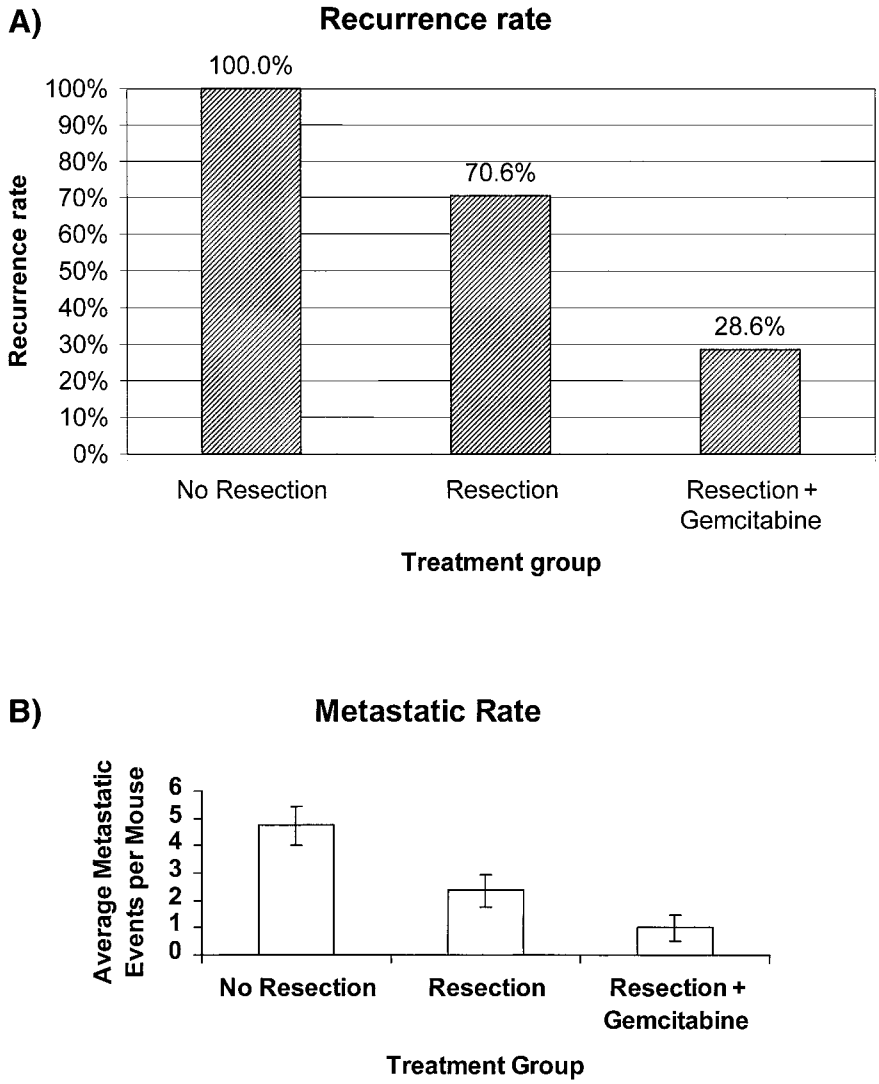

Figure 2. A) Percentage of mice in each group with recurrent tumor. All mice in the exploratory laparotomy group had tumor, whereas 70.6% of the resection group and 28.6% of the resection + gemcitabine group had tumor. B) Average metastatic events per mouse. The addition of gemcitabine reduced metastatic events by 58%.

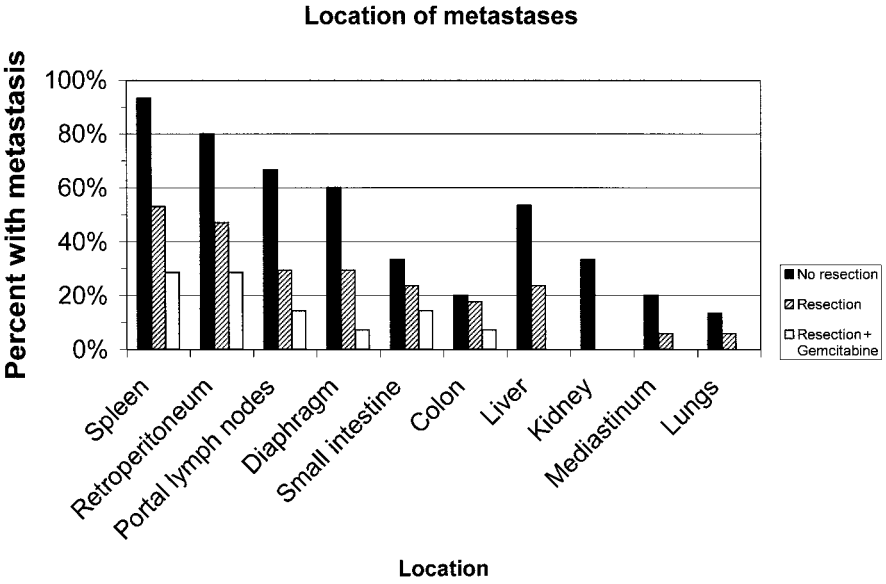

Figure 3. Location of metastases. Percentage of mice in each treatment group with recurrence in the specified location.

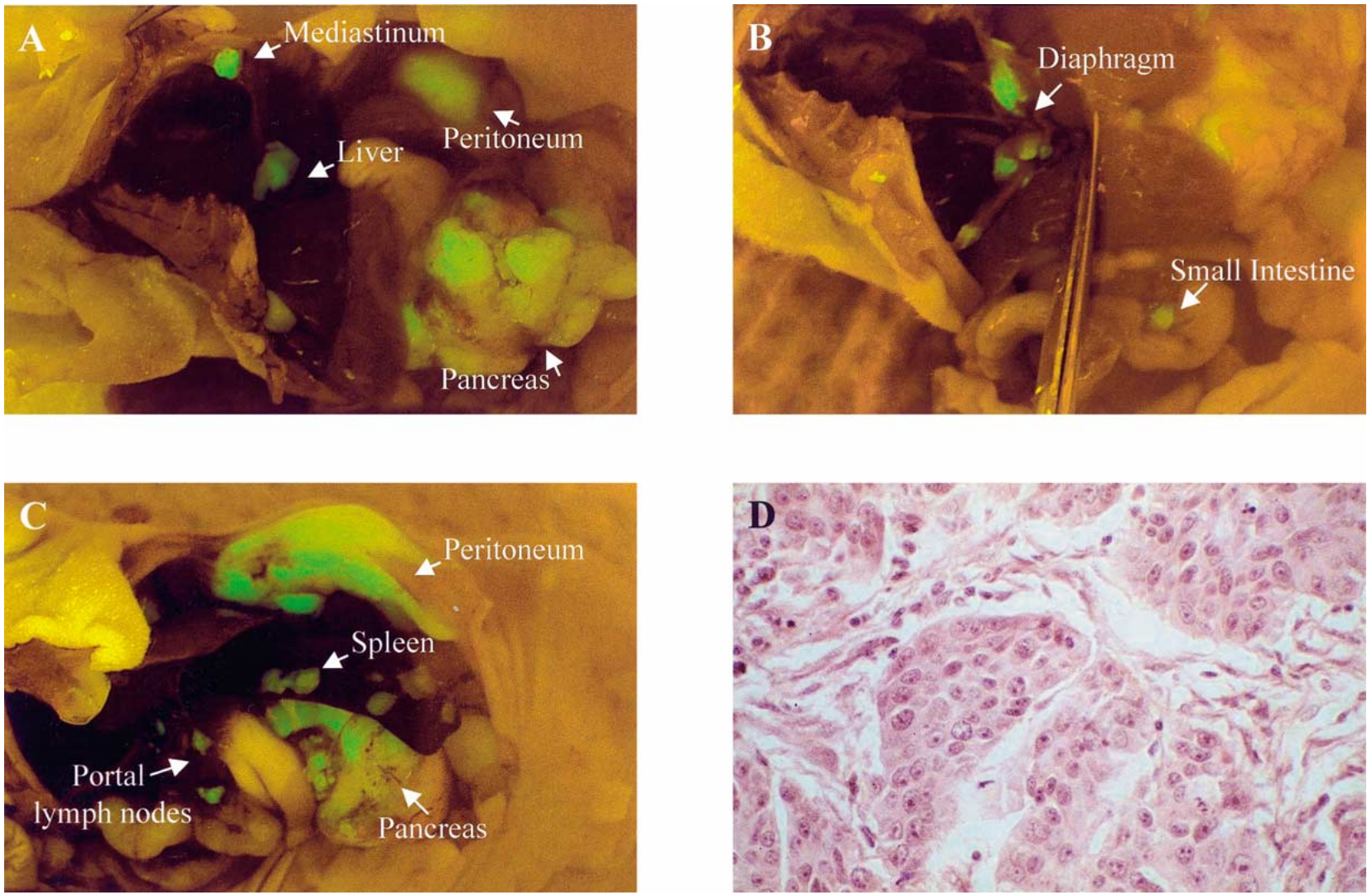

Figure 4. (A–C) Visualization of extensive metastases in three mice from Group I, including metastases to spleen, retroperitoneum, diaphragm, portal lymph nodes, small intestine, liver, and mediastinum. (D) H&E stain of the BxPC-3-GFP pancreatic adenocarcinoma.

acts to slow the rate of metastatic spread as well as reduce recurrence at the primary site, which could correlate to a prolonged disease-free survival in humans as compared to standard gemcitabine therapy [19, 20]. Furthermore, to our knowledge, our study is the first to combine gemcitabine with surgical resection of pancreatic cancer in an orthotopic model. The results of this study suggest clinical feasibility of combining surgical resection of pancreatic cancer with adjuvant gemcitabine therapy.

Acknowledgements

This study was supported in part by an American Cancer Society Institutional Research Grant and the Department of Health Services, California Cancer Research Program (97-12013).

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