Correlation of Early Recurrence With In Vitro Adenosine Triphosphate Based Chemotherapy Response Assay in Pancreas Cancer With Postoperative Gemcitabine Chemotherapy

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Introduction: Gemcitabine-based regimens represent the standard systemic first line treatment in patients after pancreatic resection. However, the clinical impact of gemcitabine varies significantly in individuals because of chemoresistance. An in vitro adenosine triphosphate based chemotherapy response assay (ATP-CRA) was designed to evaluate the sensitivity of cancer cells to various chemotherapeutic agents. This study investigated the correlation between in vitro gemcitabine sensitivity of tumor cells and early recurrence after curative resection. **Method:** From January 2007 to December 2010, the ATP-CRA for gemcitabine was tested in 64 patients surgically treated for pancreas cancer at Gangnam Severance Hospital, Seoul, Korea. We analyzed the relationship between chemosensitivity and early systemic recur-

rence in patients with pancreas cancer to predict disease-free survival (DFS) after curative resection in pancreas cancer. **Result:** The mean cell death rate (CDR) was 20.0 (± 14.5) and divided into two groups according to the mean values of the CDR. Lymphovascular invasion was more frequently shown in gemcitabine resistance group without statistical significance. In univariate and multivariate analysis, advanced tumor stage and gemcitabine sensitive group ($CDR \geq 20$) were identified as independent prognostic factors for DFS. **Conclusions:** Gemcitabine sensitivity measured by ATP-CRA was well correlated with in vivo drug responsibility to predict early recurrence following gemcitabine-based adjuvant chemotherapy in patients with pancreas cancer. *J. Clin. Lab. Anal.* **30**:804–810, 2016. © 2016 Wiley Periodicals, Inc.

Key words: pancreas cancer; postoperative adjuvant chemotherapy; chemoresistance; in vitro adenosine triphosphate based chemotherapy response assay; recurrence

INTRODUCTION

Pancreas cancer is a relatively rare disease, but it was the fourth leading cause of cancer related mortality in Korea. With the 5-year survival rate reported to be lower than 5%, the disease is associated with an extremely poor prognosis (1). Curative surgical resection is the only potentially curative treatment modality with gemcitabine-based postoperative adjuvant chemotherapy (2, 3). The clinical impact of gemcitabine varies significantly in individual tumors because of chemoresistance. So, identification of nonresponders before initiation of gemcitabine treatment may help to avoid gemcitabine-based chemotherapeutics.

An in vitro adenosine triphosphate based chemotherapy response assay (ATP-CRA) was designed to evaluate the sensitivity of cancer cells to various chemotherapeutic

agents. The ATP-CRA is a recently developed method that evaluates tumor cell viability by measuring intracellular ATP levels of drug-exposed cells and an untreated control. The clinical feasibility of ATP-CRA study has been validated in various cancers, for example those of the stomach, ovary, breast, and colorectal cancers (4–7). However, as far as we know, the clinical impact of

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ATP-CRA in clinical decision making regarding adjuvant chemotherapy in pancreas cancer has not been evaluated yet.

The aim of this study was to investigate the ability of ATP-CRA to predict disease-free survival (DFS) based on chemosensitivity to gemcitabine in pancreas cancer after curative resection patients.

MATERIALS AND METHODS

Patients and Methods

Sixty-four pancreas cancer tissues for ATP-CRA were obtained at Gangnam Severance Hospital of Yonsei University Health System from January 2007 to December 2010. Among the 64 patients, seven patients were excluded because of ATP-CRA failure and R1 (margin positive) resection patients. As a result, 57 patients were reviewed. All patients were followed up for more than 6 months and the median duration of followup was 13.8 (7.2–50.3) months. All patients received adjuvant gemcitabine chemotherapy. Patients were followed up every three month during the first 12 months, and then every 6 months beyond the first year. The Institutional Review Board of Gangnam Severance Hospital approved this study.

Adenosine triphosphate based chemotherapy response assay

ATP-CRA was performed as previously described (8). Tumor tissue specimens were taken at least 0.5 cm³ in size in the operating room and delivered to the laboratory and stored in Hank balanced salt solution (Gibco, Rockville, MD) containing 100 IU/ml penicillin (Sigma, St. Louis, MO), 100 µg/ml streptomycin (Sigma), 100 µg/ml gentamicin (Gibco), 2.5 µg/ml amphotericin B (Gibco), and 5% fetal bovine serum (Gibco). These tissue specimens were washed with ethanol, quantified, and minced before being incubated at 37°C for 12 to 16 hr with extracellular matrix-degrading cocktail enzymes containing dispase (Sigma), pronase (Sigma), and DNase (Sigma). Cells were harvested using a cell strainer (BD Falcon, Bedford, MA). To eliminate normal cells, cell suspensions were subjected to Ficoll gradient (Histopaque-1077, 1.077 g/ml, Sigma) centrifugation at 400 × *g* for 15 min. The viability of isolated cells was tested using trypan blue exclusion. Cells were then diluted to a cell concentration between 2,000 and 20,000 viable cells/100 µl for plating into a 96-well ultralow attachment microplate (Costar, Cambridge, MA) with or without anticancer drugs and cultured for 48 hr in a CO₂ incubator. The test drug concentration of gemcitabine was determined by training set experiments, which exhibited scattered distribution of cell death from each specimen. Test drug concentration value was

gemcitabine (16.9 µg/ml), which is a chemotherapeutic agent studied in several preclinical and research reports (9–11). The successful evaluation at concentration requires a minimum of 20 mg of tumor tissues. Cells from the untreated control and treated groups were lysed and the amount of ATP in the cell lysates was measured using luciferin and excessive luciferase (Roche, Mannheim, Germany) followed by flash-type luminescence measurements on a Victor 3 multilabel counter (PerkinElmer, Boston, MA). Cell death rate (CDR) was calculated as follows:

$$\text{Cell death rate (\%)} = (1 - \text{Mean of luminescence in treated well} / \text{Mean of luminescence in untreated well}) \times 100.$$

To calculate the intraassay mean coefficient of variation (CV), luminescence values of each specimen were measured three to six times in negative and positive control groups. For the purpose of quality control, a negative control group of three to six wells (only seeding medium without cell) and two positive control groups were included in the culture plate. Each positive control group composed three wells that contained the minimal (105 pg ATP) and median (280 pg ATP) amounts of ATP, as measured in 1,000 tumor cells harvested from tissue.

We then determined whether measured values at 280 pg of ATP were higher than at 105 pg of ATP. If there were contamination of microorganism, inadequate number of cells or an intraassay mean CV greater than 30, the test was considered a failure. If the measured values in the untreated control were lower than those in the positive group (105 pg of ATP), the specimen was considered to have an unacceptable viability.

Statistical Analysis

All clinicopathologic variables except age and tumor size were used as categorical variables. Differences in continuous variables between two groups were evaluated by Student's *t*-test, and differences in categorical variables were evaluated by the chi-square test. Kaplan–Meier method was used to calculate and display survival curve, and the log-rank test was performed to determine differences among all groups. The Cox proportional hazards regression method was used to determine independent prognostic factors. *P*-value less than 0.05 was considered statistically significant.

RESULTS

Clinicopathologic Characteristics

A total of 57 patients underwent R0 resection for pancreas cancer. The mean age of patients was 61.9 (±10.6)

Fig. 1. Dot plot showing CDR according to gemcitabine response assay.

years and consisted of 27 men and 30 women. Of the 57 patients, 13 underwent distal pancreatectomy, 6 underwent pancreaticoduodenectomy, 34 underwent pylorus-preserving pancreaticoduodenectomy, and 4 underwent total pancreatectomy. All patients underwent dissection of the lymph nodes in the hepatoduodenal ligament, common hepatic artery, and celiac axis. According to the seventh AJCC classification, 1 patient was stage IA, 3 patients were stage IB, 14 patients were stage IIA, 35 patients were stage IIB, and 4 patients were stage III, respectively. All patients received adjuvant chemotherapy with six cycles of gemcitabine every 4 weeks. Each chemotherapy cycle consisted of three weekly infusions of gemcitabine 1000 mg/m² given by intravenous infusion during a 30-min period, followed by a 1-week pause.

Adenosine triphosphate based chemotherapy response assay

Sixty-four chemosensitivity tests were conducted. Three patients failed because of contamination and inadequate samples. Therefore, ATP-CRA data were successfully generated from 95.3% of samples tested for gemcitabine. The mean CDR was 20.0 (± 14.5) and we divided into two groups according to the mean values of the CDR; low <20 or high ≥ 20 . Thirty-three of 57 patients were

categorized into the gemcitabine resistance (<20), and 24 of 57 patients were into the gemcitabine sensitive group (≥ 20 , Fig. 1).

Analysis of clinicopathologic features and ATP-CRA

The clinicopathologic features and ATP-CRA status of patients in the study group are summarized in Table 1. Lymphovascular invasion was more frequently shown in gemcitabine resistance group, without statistical significance. Patient age and sex, tumor size, histologic grade, tumor stage, and lymph node metastasis did not correlate with ATP-CRA status (Table 1).

DFS and ATP-CRA

In these 57 patients, the 1- and 3-year DFS rates were 38.7% and 4.4%, respectively. In univariate analysis, lymph node metastasis, advanced tumor stage, and gemcitabine resistance group were significant poor prognostic factors (Table 2). In multivariate analysis, advanced tumor stage and gemcitabine resistance group were identified as independent prognostic factors for DFS (Table 3).

For patients with gemcitabine resistance, the 1- and 3-year DFS rates were 25.3% and 3.6 %, respectively. In patients with gemcitabine sensitive group, the 1- and

TABLE 1. Clinicopathologic Characteristics According to ATP-CRA

	Gemcitabine resistance group (<20) N = 33	Gemcitabine-sensitive group (≥20) N = 24	P-value
Age (mean ± SD, years)	62.5 ± 9.4	61.2 ± 12.3	0.648
Sex			0.843
Male	16	11	
Female	17	13	
Operation name			0.071
PD/PPPD*	27	13	
Distal pancreatectomy	5	8	
Total pancreatectomy	1	3	
Combine resection			0.561
No	27	21	
Yes	6	3	
Tumor size (mean ± SD, cm)	3.5 ± 2.2	3.4 ± 1.5	0.889
Histologic grade			0.489
Well differentiation	10	4	
Moderate differentiation	20	17	
Poor differentiation	3	3	
Depth of invasion			0.394
T1	1	0	
T2	1	3	
T3	28	20	
T4	3	1	
Lymph node metastasis			0.569
No	10	9	
Yes	23	15	
Perineural invasion			0.423
No	10	5	
Yes	23	19	
Lymphovascular invasion			0.051
No	21	9	
Yes	12	15	
Tumor stage			0.668
IA	1	0	
IB	1	2	
IIA	7	7	
IIB	21	14	
III	3	1	

PD/PPPD*, pylorus-preserving pancreaticoduodenectomy.

3-year DFS rates were 57.4% and 0.0 %, respectively (Fig. 2).

DISCUSSION

This study evaluated predictive value of ATP-CRA on the outcome of pancreas cancer and, to our knowledge, it is the first study to demonstrate a significant association between ATP-CRA results and DFS in patient who underwent radical resection and were treated with gemcitabine in the adjuvant setting.

TABLE 2. Univariate Prognostic Factors of DFS in Curative Resection of Pancreas Cancer

	DFS (%)		P-value
	1 year	3 year	
Age			0.329
<60 years	34.2	11.4	
≥60 years	40.9	0.0	
Sex			0.853
Male	40.8	0.0	
Female	36.7	6.9	
Operation Name			0.623
PD/PPPD	31.0	11.6	
Distal pancreatectomy	53.8	0.0	
Total pancreatectomy	75.0	0.0	
Combine resection			0.075
No	42.8	5.2	
Yes	13.3	0.0	
Tumor size			0.701
<3 cm	27.3	8.2	
≥3 cm	46.3	0.0	
Histologic grade			0.344
Well differentiation	64.3	18.4	
Moderate differentiation	32.1	0.0	
Poor differentiation	16.7	0.0	
Depth of invasion			0.228
T1	100	100	
T2	50.0	25.0	
T3	37.5	0.0	
T4	25.0	25.0	
Lymph node metastasis			0.047
No	56.4	0.0	
Yes	32.8	3.8	
Perineural invasion			0.595
No	40.0	13.3	
Yes	38.0	0.0	
Lymphovascular invasion			0.881
No	42.7	15.1	
Yes	35.2	0.0	
Tumor stage			0.037
IA	100	100	
IB	66.7	33.3	
IIA	62.3	0.0	
IIB	26.8	0.0	
III	25.0	0.0	
Chemotherapy response assay			0.017
Gemcitabine resistance (CDR < 20)	25.3	3.6	
Gemcitabine-sensitive (CDR ≥ 20)	57.4	0.0	

PD/PPPD*, pylorus-preserving pancreaticoduodenectomy.

A recent study supported gemcitabine-based chemotherapy as the standard treatment for pancreas cancer, and it is generally recommended for first line treatment (3). Oettle et al. (3) reported that in the advanced stage (stage 3/4), median DFS was as short as 6.4 months in the control group, while median DFS nearly doubled to reach 12.1 months after treatment with gemcitabine. They concluded that the beneficial effect of

TABLE 3. Multivariate Prognostic Factors of DFS in Curative Resection of Pancreas Cancer

Variables	P-value	Odds ratio	Confidence interval (95%)	
			Lower	Upper
Advance tumor stage	0.006	0.424	0.326	0.761
Gemcitabine resistance group	0.009	0.419	0.219	0.802

adjuvant gemcitabine offers chance for prolonged DFS in patients undergoing curative resection for pancreas cancer. Therefore, postoperative adjuvant treatment is recommended as gemcitabine or 5-FU/leucovorin treatment in all pancreas cancer patients according to NCCN guidelines. However, identification of patients who have clinical benefit in the adjuvant chemotherapy is a valuable issue for the tailored therapy in pancreas cancer because only small patients have DFS period

at 12 months after surgery (3). Because gemcitabine remains the backbone of systemic therapy for pancreatic cancer, we evaluated in vitro chemosensitivity testing of potential relevance to gemcitabine sensitivity. In vitro chemosensitivity testing of cells obtained from tumor tissues has better economic effect and reduces toxic side effects of the therapy (12). In vitro chemosensitivity testing method is generally divided into cell proliferation and cell death assay. ATP-CRA assay is a cell death assay and designed to evaluate evaluates tumor cell viability by measuring intracellular ATP levels of drug-exposed cells and an untreated control. ATP assay reported high success rate and requires only a small number of tumor cells (13). In this study, we tested a gemcitabine in tumor cells isolated form clinical samples of solid tumors. This chemosensitivity testing results showed that success rate of isolated pancreas cancer cells to gemcitabine was 95.3%, which was a level similar to previous reports (13, 14). Thus, our study confirms that ATP-CRA is a stable in vitro chemosensitivity assay for pancreas cancer patients. This high assessability rate may be related to the

Fig. 2. Disease-free survival rate according to chemotherapy response assay results.

rapid and exact sampling of the cancer tissue by surgeon performed immediately after a surgical resection. Our results showed that lymphovascular invasion was more frequently observed in the gemcitabine resistance group, without statistical significance. But, Uematsu et al. (15) reported lymphovascular invasion is one of the important factors to predict chemotherapy efficacy in breast cancer. Therefore, further studies will be needed to more precisely define the relation between lymphovascular invasion and chemoresistance.

We found that patients with gemcitabine resistance group had a shorter DFS when compared with patients with gemcitabine-sensitive group. In our multivariate analysis, gemcitabine resistance group was significant independent marker of DFS.

These results are concordant with previous reports of the prognostic value of ATP-CRA chemosensitivity in unresectable pancreas cancer (16). Gemcitabine resistance group in advanced/metastatic pancreas cancer treated with gemcitabine was associated with a shorter progression-free survival compared with patients for whom gemcitabine sensitive group.

This study has limitations. First, we studied the ATP-CRA only for single agents, the effect of combining anticancer drugs could not be investigated. Single-agent chemotherapy with gemcitabine is considered by many oncologists to represent the “gold standard” for adjuvant chemotherapy after curative resection for pancreas cancer. However, after adjuvant chemotherapy failure, several agents in combination are administrated. Drug interactions may be more important for the clinical utility of a drug combination than isolated activity of its components. Some papers reported that combinations of gemcitabine and platinum or mitoxantrone and paclitaxel are good examples because these combinations display features of true clinical synergism, which may be related to resistance-modulating effects (17,18). Thus, it is of particular importance that drug combinations be assayed when the patients are planned for treatment with combination chemotherapy.

Second, in the ATP-CRA, tumor cells and stromal cells are separated to obtain a single-cell suspension. Thereby, cell-to-cell interactions, between tumor cells and surrounding stromal cells, are disrupted. In cancer, extracellular matrix proteins play important roles in tumor acquisition of resistance to anticancer drugs. Thus, chemoresistance variations between in vitro and in vivo conditions required further evaluation. Nevertheless, if resistance is present in vitro assay, it would probably result in clinical treatment failure.

Third, our study had small number of patients. With respect to the small number of patients, validation of our outcome using independent cohort was impossible. Therefore, large-scale trials will be necessary to define

the clinical value of CRA chemosensitivity for pancreatic cancer.

In conclusions, this is the first study to apply in vitro ATP-CRA as a predictor of chemoresponse for pancreas cancer in adjuvant chemotherapy setting. In vitro ATP-CRA might be a useful assay to predict the patients who benefit from gemcitabine-based adjuvant chemotherapy after curative resection of pancreas cancer. Further randomized controlled trials are needed to confirm the advantage of ATP-CRA to predict the response to adjuvant chemotherapy in pancreas cancer

CONFLICT OF INTEREST

The authors have nothing to disclose.

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