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Gene Expression Levels as Predictive Markers of Outcome in Pancreatic Cancer after Gemcitabine-Based Adjuvant Chemotherapy[1,2]

Hayato Fujita*, Kenoki Ohuchida*,†, Kazuhiro Mizumoto*,‡, Soichi Itaba§, Tetsuhide Ito§, Kohei Nakata*,¶, Jun Yu*, Tadashi Kayashima*, Ryota Souzaki#, Tatsuro Tajiri#, Tatsuya Manabe*, Takao Ohtsuka* and Masao Tanaka*

*Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; †Department of Advanced Medical Initiatives, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; ‡Kyushu University Hospital Cancer Center, Fukuoka, Japan; §Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; ¶Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; #Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Abstract

BACKGROUND AND AIMS: The standard palliative chemotherapy for pancreatic ductal adenocarcinoma (PDAC) is gemcitabine-based chemotherapy; however, PDAC still presents a major therapeutic challenge. The aims of this study were to investigate the expression pattern of genes involved in gemcitabine sensitivity in resected PDAC tissues and to determine correlations of gene expression with treatment outcome. *MATERIALS AND METHODS:* We obtained formalin-fixed paraffin-embedded (FFPE) tissue samples from 70 patients with PDAC. Of the 70 patients, 40 received gemcitabine-based adjuvant chemotherapy (AC). We measured *hENT1*, *dCK*, *CDA*, *RRM1*, and *RRM2* messenger RNA (mRNA) levels by quantitative real-time reverse transcription–polymerase chain reaction and determined the combined score (GEM score), based on the expression levels of *hENT1*, *dCK*, *RRM1*, and *RRM2*, to investigate the association with survival time. By determining the expression levels of these genes in endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) cytologic specimens, we investigated the feasibility of individualized chemotherapy. *RESULTS:* High *dCK* ($P = .0067$), low *RRM2* ($P = .003$), and high GEM score ($P = .0003$) groups had a significantly longer disease-free survival in the gemcitabine-treated group. A low GEM score (<2) was an independent predictive marker for poor outcome to gemcitabine-based AC as shown by multivariate analysis ($P = .0081$). Altered expression levels of these genes were distinguishable in microdissected neoplastic cells from EUS-FNA cytologic specimens. *CONCLUSIONS:* Quantitative analyses of *hENT1*, *dCK*, *RRM1*, and *RRM2* mRNA levels using FFPE tissue samples and microdissected neoplastic cells from EUS-FNA cytologic specimens may be useful in predicting the gemcitabine sensitivity of patients with PDAC.

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Abbreviations: 5′-NT, 5′-nucleotidase; AC, adjuvant chemotherapy; CDA, cytidine deaminase; dCK, deoxycytidine kinase; DFS, disease-free survival; EUS-FNA, endoscopic ultrasound-guided fine needle aspiration; FFPE, formalin-fixed paraffin-embedded; hENT1, human equilibrative nucleoside transporter 1; IC_{50}, 50% inhibitory concentration; NSCLC, non–small cell lung cancer; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; qRT-PCR, quantitative real-time reverse transcription–polymerase chain reaction; RR, ribonucleotide reductase; UICC, Union Internationale Contre le Cancer and the American Joint Committee on Cancer; WCP, whole cell pellet

Address all correspondence to: Kenoki Ohuchida or Kazuhiro Mizumoto, Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan. E-mail: kenoki@med.kyushu-u.ac.jp or mizumoto@med.kyushu-u.ac.jp

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[2]This article refers to supplementary materials, which are designated by Tables W1 to W4 and Figure W1 and are available online at www.neoplasia.com.
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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal and aggressive human malignancies, being the fourth leading cause of tumor-related deaths in the industrialized world [1,2]. Most patients with PDAC have poor outcomes because of the aggressive biology of the tumor and the difficulties of early diagnosis because of a lack of early disease-specific signs and symptoms. Only 10% to 20% of patients with PDAC are candidates for curative resection [3], and even if the curative resection is performed, the postoperative 5-year survival rate is only 15% to 25% because of a high recurrence rate [4,5]. Although two recent randomized clinical phase 3 trials of adjuvant chemotherapy (AC) for PDAC showed significant increases in overall survival (OS) and disease-free survival (DFS) [6,7], there remain a substantial subset of cases in which AC efficacy is limited and insufficient. Recent studies have revealed that altered gene expression can at least partly explain responses and toxicity of cytotoxic agents [8]. To improve the prognosis of patients with PDAC, a helpful strategy would be to select subjects who are likely to respond to treatment based on gene expression profiles of the individual's own cancer tissues.

Gemcitabine (difluorodeoxycytidine; dFdC) is a deoxycytidine analog that has broad antitumor activity in various solid tumors, including pancreatic cancer [7] and non–small cell lung cancer (NSCLC) [9]. The drug is a prodrug that requires cellular uptake and intracellular phosphorylation to produce active diphosphate (dFdCDP) and triphosphate (dFdCTP). These phosphorylated forms function by inhibiting ribonucleotide reductase (RR) and DNA synthesis [10]. Gemcitabine is transported into cells predominantly by human equilibrative nucleoside transporter 1 (hENT1) [11]. A deficiency in hENT1 activity conferred high-level resistance to the toxicity of gemcitabine [12], and patients with PDAC that have detectable hENT1 or high *hENT1* gene expression have significantly prolonged survival after gemcitabine chemotherapy [13,14]. After cellular entry, gemcitabine must be phosphorylated by deoxycytidine kinase (dCK), which is a rate-limiting step. We previously demonstrated that down-regulation of *dCK* specifically enhanced acquired resistance to gemcitabine in pancreatic cancer cells [15], whereas transfection of wild-type *dCK* restored sensitivity to the drug [16]. Conversely, active metabolites of gemcitabine are reduced by 5′-nucleotidase (5′-NT), and gemcitabine itself is inactivated by cytidine deaminase (CDA). High levels of these catabolic enzymes are associated with resistance to the drug [17,18]. dFdCTP inhibits DNA synthesis by being incorporated into the DNA strand, but in addition, dFdCDP potently inhibits RR, resulting in a decrease of competing deoxyribonucleotide pools necessary for DNA synthesis [19]. RR is a dimeric enzyme composed of regulatory subunit M1 and catalytic subunit M2. Recurrent PDAC patients with high levels of *RRM1* expression had poor survival rates after gemcitabine treatment [20], and NSCLC patients with low levels of *RRM1* expression significantly benefited from gemcitabine/cisplatin neoadjuvant chemotherapy [21]. Moreover, *RRM2* gene silencing by RNA interference is an effective therapeutic adjunct to gemcitabine treatment [22]. These data suggest that the genes encoding proteins involved in the transport and metabolism of gemcitabine and in the metabolism of targets can be potential candidates to predict sensitivity to gemcitabine.

To develop individualized chemotherapy, the characterization of genes associated with tumor sensitivity or resistance to antitumor agents using cancer tissues from individuals plays a critical role in the selection of preferable treatments. In the current study, we investigated the correlation between the expression of genes involved in cellular uptake and metabolism of gemcitabine and the treatment outcome of patients with PDAC who underwent gemcitabine-based AC or no AC. Furthermore, to investigate the feasibility of individualized chemotherapy for patients with PDAC, even when the tumor is unresectable, we quantified the expression of genes in cytologic specimens obtained from endoscopic ultrasound-guided fine needle aspiration (EUS-FNA).

Materials and Methods

Cell Lines and Establishment of Gemcitabine-Resistant Cells

We used two pancreatic cancer cell lines, SUIT-2 (generously provided by Dr H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan) and Capan-1 (American Type Culture Collection, Manassas, VA). Gemcitabine-resistant Capan-1-GR and SUIT-2-GR cells were generated by exposing to gradually increasing concentrations of gemcitabine as described previously [15]. Cells were maintained as described previously [23].

Propidium Iodide Assay

To calculate the 50% inhibitory concentration (IC_{50}) of each cell line when exposed to gemcitabine, cells were seeded in 24-well plates (Becton Dickinson Labware, Bedford, MA) at a density of 2×10^4 per well, using cell numbers previously counted using a particle distribution analyzer CDA 500 (Sysmex, Kobe, Japan). Several different concentrations of gemcitabine (Wako, Osaka, Japan) were added 24 hours after seeding. Cell populations were evaluated by measuring the fluorescence intensity of propidium iodide after a further incubation for 72 hours, as described previously [24].

Patients and Pancreatic Tissues

Our study subjects consisted of 70 patients including 40 patients who received gemcitabine-based AC (GEM group) and 30 patients who received no AC (non-AC group) after pancreatic resection for PDAC at the Department of Surgery and Oncology, Kyushu University Hospital (Fukuoka, Japan) from 1992 to 2007. Although there were 48 patients who received gemcitabine-based AC, eight patients were excluded because they did not receive adequate AC. The GEM group patients (n = 40) received gemcitabine-based AC, consisting of two or more cycles of 1000 mg/m^2 per day of gemcitabine on days 1, 8, and 15 every 28 days, or three or more cycles of 1000 mg/m^2 per day of gemcitabine on days 1 and 8 every 21 days. The patients were 42 men and 28 women with a median age of 65 years (range, 36-86 years). We recommended that patients have follow-up visits every 3 months for 2 years, then visits every 6 months for 3 years, and then annual visits. DFS was defined as the time from the date of pancreatic resection to the date of local or distant recurrence. The date of recurrence was defined as the date of the first subjective symptom heralding relapse, or the date of documentation of recurrent disease, independent of site, as assessed by diagnostic imaging techniques (whichever occurred first). Data for patients without recurrence were censored at the time of the last follow-up visit. OS was measured from the date of pancreatic resection to the date of death. Fifty-seven patients died during follow-up, and the other patients were censored at the time of the last follow-up visit. Data were analyzed in December 2009, and follow-up data from all cases were available. The median observation time for DFS was 8.0 months (range, 0.5-114 months) and that for OS was 15.7 months (range, 0.5-114 months). The clinicopathologic characteristics of the tumors collected from a total of 70 patients are noted in Table W1.