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Precision design of nanomedicines to restore gemcitabine chemosensitivity for personalized pancreatic ductal adenocarcinoma treatment

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ABSTRACT

Low chemosensitivity considerably restricts the therapeutic efficacy of gemcitabine (GEM) in pancreatic cancer treatment. Using immunohistochemical evaluation, we investigated that decreased expression of human equilibrative nucleoside transporter-1 (hENT1, which is the major GEM transporter across cell membranes) and increased expression of ribonucleotide reductase subunit 2 (RRM2, which decreases the cytotoxicity of GEM) was associated with low GEM chemosensitivity. To solve these problems, we employed a nanomedicine-based formulation of cationic liposomes for co-delivery of GEM along with siRNA targeting RRM2. Due to the specific endocytic uptake mechanism of nanocarriers and gene-silencing effect of RRM2 siRNA, this nanomedicine formulation significantly increased GEM chemosensitivity in tumor models of genetically engineered Panc1 cells with low hENT1 or high RRM2 expression. Moreover, in a series of patient-derived cancer cells, we demonstrated that the therapeutic benefits of the nanomedicine formulations were associated with the expression levels of hENT1 and RRM2. In summary, we found that the essential factors of GEM chemosensitivity were the expression levels of hENT1 and RRM2, and synthesized nanoformulations can overcome these problems. This unique design of nanomedicine not only provides a universal platform to enhance chemosensitivity but also contributes to the precision design and personalized treatment in nanomedicine.

1. Introduction

Ever since the effective use of aminopterin and nitrogen mustards for cancer treatment in the mid 20th century [1,2], chemotherapy has profoundly influenced the survival of many cancer patients. Despite the successful development of chemotherapeutic agents, one of major hurdles for successful chemotherapy is drug resistance [3]. Resistance after initial drug treatment leads to unsatisfactory chemotherapeutic effects in cancer patients, especially in individuals with cancers of a low resectable rate, such as pancreatic ductal adenocarcinoma (PDAC).

Due to the difficulty of early diagnosis, only 20% of patients with pancreatic cancer are considered surgically resectable [4]. For the other 80% of patients with advanced pancreatic cancer, radiotherapy [5] and recently developed targeted therapy [6] have limited survival benefit; the main treatment scheme remains to be

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chemotherapy. The median overall survival of patients with metastatic pancreatic cancer is fewer than 7 months, even when patients are treated with the current first-line regimen, gemcitabine (GEM, 2′-deoxy-2′,2′-difluorocytidine, dFdC) [6]. The objective response rate was only 9.4% after GEM single agent treatment in pancreatic cancer patients [6], which is attributed to severe drug resistance against GEM in the majority of patients. Although there are many causes of drug resistance in clinic, including limited vascular accessibility and dense extracellular matrix as drug delivery barriers in the tumor microenvironment, the major cause of drug resistance is the low chemosensitivity of cancer cells *per se* [7–9].

The process of cellular uptake and intracellular metabolism of GEM in cancer cells is complex, and many factors affect GEM cytotoxicity (Fig. S1) [10]. As a pro-drug with high hydrophilicity, GEM penetration across the cell membrane depends on an appropriate transporter. In human cancer cells, human equilibrative nucleoside transporter-1 (hENT1, GenBank accession no. 2030) was identified as the major GEM transporter across cell membranes [11,12]. Once inside the cell, GEM must be converted to its diphosphate (dFdCDP) and triphosphate (dFdCTP) forms. During this transformation, deoxycytidine kinase (dCK, GenBank accession no. 1633) is the rate-limiting enzyme [13–15]. dFdCTP inhibits DNA synthesis by being incorporated into DNA, where it leads to masked chain termination and subsequent apoptosis. Also, dFdCDP can irreversibly inactivate ribonucleotide reductase (RR), which catalyzes the conversion of ribonucleoside diphosphates (NDP) to deoxyribonucleoside diphosphates (dNDP), which is essential for DNA replication [16]. Moreover, it has been reported that overexpression of RR induces high levels of dNTP pools, which can competitively inhibit the incorporation of dFdCTP into DNA, thereby decreasing the cytotoxicity of GEM [17]. Human RR is a dimeric enzyme comprising two subunits, M1 (RRM1, GenBank accession no. 6240) and M2 (RRM2, GenBank accession no. 6241) [18,19]. Although the potential correlation between the expression levels of the four essential proteins in GEM metabolism (hENT1, dCK, RRM1, and RRM2) and GEM chemosensitivity in both pancreatic cancer cells and clinical studies have been reported [20–26], there is still no consensus on the key factors involved in GEM chemosensitivity at present.

Nanocarrier-based drug delivery systems have been devised and evaluated for their ability to deliver therapeutic cargoes (such as drugs and/or siRNA) to overcome drug resistance [27–31]. The solutions based on nanomedicines have been applied as a co-delivery of multiple drugs and/or chemosensitizers to increase local drug concentrations due to passive and/or active tumor target effects. To maximize the clinical benefit of nanomedicines, an in-depth understanding of the mechanisms by which tumors resist drugs is critical for the customized design of nanocarriers and identification of the patient subpopulations responding to a given nanomedicine. Herein, we describe a precise design to increase GEM chemosensitivity in pancreatic cancer. Our strategy began with a rigorous statistical analysis of clinical data to determine that the key factors responsible for low GEM chemosensitivity in North China were low hENT1 and high RRM2 expression, followed by the design of functional nanocarriers directly address the clinical discovery. The delivery of GEM by nanocarriers and endocytosis into cancer cells could overcome the low chemosensitivity caused by low hENT1 expression. In addition, the co-delivery with siRNA against RRM2 would enhance the chemosensitivity of GEM in cancer cells with high RRM2 expression. Next, genetically engineered pancreatic cancer cell lines with different expression levels of those key factors were utilized to assess the therapeutic efficacy of the nanomedicine. Finally, the antitumor efficacy of GEM and the nanomedicine formulations were evaluated in primary patient-derived pancreatic cancer cells. The different levels of therapeutic benefit revealed the importance and necessity of personalized application of nanomedicine.

2. Materials and methods

2.1. Patient cohort and clinical treatment

The use of human samples was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital and followed the ethical guideline. We obtained the informed consent from all subjects. We retrospectively enrolled all patients who received a radical resection with a pathologically confirmed diagnosis of pancreatic ductal adenocarcinoma and were treated at Tianjin Cancer Hospital from December 2009 to January 2013. The follow-ups for final analysis ended on June 30, 2015. Patients with at least one of the following conditions were excluded: (1) patients who received neoadjuvant chemotherapy, chemoradiotherapy, or non-GEM-based chemotherapy; (2) patients with macroscopically incomplete resection; (3) patients with a history of another major cancer; (4) patients who died within one month after the operation or due to non-cancer related causes. The patients received at least three cycles of GEM-based chemotherapy after operation. GEM was delivered by a 30-min intravenous infusion at a dose of 1000 mg per square meter of body surface area weekly for two weeks followed, by one week intervals, then for two weeks in a subsequent three-week course.

2.2. Immunohistochemistry

For IHC analysis of tissue microarray, anti-hENT1 mouse monoclonal antibody (sc-377283, 1:100, Santa Cruz, USA), anti-dCK rabbit polyclonal antibody (ab151966, 1:500, Abcam, UK), anti-RRM1 rabbit monoclonal antibody (ab133690, 1:350, Abcam, UK), and anti-RRM2 mouse monoclonal antibody (ab57653, 1:500, Abcam, UK) were used to determine the levels of protein expression of hENT1, dCK, RRM1 and RRM2, respectively. IHC slides were independently graded by two pathologists, who were blinded to patient outcomes. Discordant cases were assessed by a third pathologist, and a consensus was reached.

Membrane and cytoplasmic staining for hENT1 were regarded as positive. Cytoplasmic staining for dCK, RRM1 and RRM2 was regarded as positive. Immunoreactivity was scored semi-quantitatively according to the intensity and extent of tumor cell staining. The intensity of tumor cells staining was scored as 0 = negative, 1 = low, 2 = medium and 3 = high. The extent of staining was scored as 0 = 0%–5% staining, 1 = 5%–25% staining, 2 = 26%–50% staining and 3 = 51%–100% staining. The final score was determined by multiplying the scores of intensity with the extent of staining, in the range of 0–9. Final scores of less than 1 were considered negative (−), 1–2 as low staining (+), 3–4 as medium staining (++) and 6–9 as high staining (+++).

2.3. Synthesis of nanoformulations

The DOTAP-based cationic liposome nanoparticles (approved by the US Food and Drug Administration for clinical trials, NCT00059605) were prepared by a lipid film method. Briefly, a 15 μmol lipid mixture of DOTAP (LP-R4-117, Ruixi Biological Technology Co., China), dioleoyl-phosphatidylethanolamine (DOPE, LP-R4-069, Ruixi Biological Technology Co., China), cholesterol (Chol, 121530, JK Chemical, China) and distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (DSPE-mPEG2000, LP-R4-039, Ruixi Biological Technology Co., China) at a molar ratio of 8:3:8:1 was dissolved in 10 ml