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Gene expression profiling for analysis acquired oxaliplatin resistant factors in human gastric carcinoma TSGH-S3 cells: The role of IL-6 signaling and Nrf2/AKR1C axis identification

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ABSTRACT

Oxaliplatin treatment is a mainstay of treatment for advanced gastrointestinal tract cancer, but the underlying mechanisms of acquired oxaliplatin resistance remain largely obscured. We previously demonstrated that increased DNA repair capacity and copper-transporting ATPase 1 (ATP7A) level contributed to oxaliplatin resistance in the human gastric carcinoma cell line TSGH-S3 (S3). In the present study, we applied gene array technology to identify additional resistance factors in S3 cells. We found that interleukin-6 (IL-6), aldo-keto reductase 1C1 (AKR1C1), and AKR1C3 are the top 3 upregulated genes in S3 cells when compared with parent TSGH cells. Despite a higher level of endogenous IL-6 in S3, IL-6 receptor (IR-6R, gp-80, and gp-130) levels were similar between TSGH and S3 cells. The addition of exogenous IL-6, IL-6 targeted siRNA, or neutralizing antibodies neither affected Stat3 activation, a downstream target of IL-6, nor changed oxaliplatin sensitivity in S3 cells. However, manipulation of AKR1C activity with siRNA or AKR1C inhibitors significantly reversed oxaliplatin resistance. AKR1Cs are classical antioxidant response element (ARE) genes that can be transcriptionally upregulated by nuclear factor erythroid 2-related factor 2 (Nrf2). Knockdown of Nrf2 not only decreased the levels of AKR1C1, AKR1C2, and AKR1C3 mRNA and protein but also reversed oxaliplatin resistance in S3 cells. Taken together, these results suggest that activation of the Nrf2/AKR1C axis may contribute to oxaliplatin resistance in S3 cells but that the IL-6 signaling pathway did not contribute to resistance. Manipulation of Nrf2/AKR1Cs activity may be useful for management of oxaliplatin-refractory gastric cancers.

Abbreviations: AKR1Cs, aldo-keto reductase family members (e.g. AKR1C1, AKR1C2, or AKR1C3); ARE, antioxidant response element; cisplatin, *cis*-diamminedichloroplatinum(II) (CDDP); ERCC-1, excision repair cross-complementing-1; ERK, extracellular signal-related kinase; FFA, flufenamic acid; GCLC, γ-glutamyl cysteine synthetase catalytic subunit; GCLM, γ-glutamyl cysteine synthetase modifier subunit; GI, gastrointestinal tract; GR, glutathione reductase; GST, glutathione S-transferase; HIF-1α, hypoxia-inducible factor-1α; HO-1, heme oxygenase-1; IL-6, interleukin-6; IL -6R/gp80, IL-6 receptor α subunit; IL -6R/gp130, IL-6 receptor β subunit; JAK1, Janus kinase 1; JAK2, Janus kinase 2; MAPK, mitogen-activated protein kinase; MCA, α-methylcinnamic acid; NQO-1, NAD(P)H:quinone oxidoreductase; Nrf2, nuclear factor erythroid 2-related factor 2; PI3K, phosphatidylinositol 3-kinase; PPTH, phenolphthalein; S3, an oxaliplatin-resistant human gastric carcinoma cell line (TSGH-S3); STAT3, signal transducer and activator of transcription 3; TR, thioredoxin reductase; TYK2, tyrosine kinase 2.

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1. Introduction

Oxaliplatin is a third generation platinum compound, which when used in combination with other cytotoxic agents such as 5-fluorouracil and folinic acid has significant activity in a wide variety of gastrointestinal tract (GI) cancers, including colorectal, gastric, pancreatic, and hepatobiliary malignancies [1–4]. It induces cytotoxicity through the formation of platinum-DNA adducts, which inhibit DNA replication and lead to cell death [5]. Despite its impressive clinical efficacy, virtually all patients with advanced GI cancers develop oxaliplatin-resistant tumors within months of therapy. Oxaliplatin-resistant cells are characterized by defects in cellular oxaliplatin accumulation and impaired DNA adduct formation [6]. In addition, autophagy activation, abnormalities in the apoptotic pathway, and hypoxia with activation of hypoxia-inducible factor-1α (HIF-1α) may also play a role in cellular resistance to oxaliplatin [7–9].

In spite of this information, the mechanisms underlying acquired oxaliplatin resistance are less well understood compared to those of the parental compound, *cis*-diamminedichloroplatinum(II) (cisplatin, CDDP) [5,10,11]. For example, increased expression of excision repair cross-complementing-1 (ERCC-1) as a predictor of poor clinical outcome to cisplatin treatment exemplifies the success of studies focused on the cellular pharmacology of cisplatin resistance [12,13]. By contrast, studies of the mechanisms of oxaliplatin resistance have failed to identify prognostic markers that could help predict drug response in individual patients. Therefore, a major goal of research in the field is to identify the mechanisms that mediate oxaliplatin resistance with the aim of either preventing or overcoming this problem. This may facilitate individualized "tailor-made" therapy, as novel markers found during such research could be used as predictors of clinical efficacy of oxaliplatin treatment.

To dissect the mechanisms underlying oxaliplatin resistance, we established an oxaliplatin-resistant cell line, TSGH-S3 (S3), from human gastric adenocarcinoma TSGH cells. We found that S3 cells expressed a higher level of the copper efflux transporter – copper-transporting ATPase 1 (ATP7A), retained less oxaliplatin in the cytoplasm, were less susceptible to DNA adduct formation, and had enhanced DNA repair activity in comparison with parental TSGH cells [14]. These mechanisms were believed to contribute to, at least in part, the development of oxaliplatin resistance. In our current study, we applied global transcriptional analysis using gene array technology to investigate the difference between the oxaliplatin-resistant S3 cell line and the parental TSGH cells. Our aim was to further elucidate the mechanisms and molecular targets involved in acquired oxaliplatin resistance of gastric cancers.

2. Materials and methods

2.1. Reagents and supplies

The platinum compounds oxaliplatin and cisplatin were obtained from TTY Biopharm (Taipei, Taiwan) and Sigma–Aldrich (St. Louis, MO, USA), respectively. Ruxolitinib was a kind gift from Novartis Pharma (Basel, Switzerland). WP1066 and S3I-201 were purchased from Calbiochem (La Jolla, CA, USA). PD98059 and U0126 were purchased from Cell Signaling Technology (Danvers, MA, USA). SP60125, flufenamic acid (FFA), α-methylcinnamic acid (MCA), and phenolphthalein (PPTH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human recombinant interleukin-6 (IL-6) and IL-6 antibody (IL-6 Ab) were obtained from R&D Systems (Minneapolis, MN, USA). All other chemicals were obtained from E. Merck Co. (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, USA) and were of standard analytic grade or higher.

2.2. Cell culture

The human gastric adenocarcinoma cell line TSGH was cultured in minimal essential medium supplemented with 5% fetal bovine serum and 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamate in a 5% CO_2 humidified atmosphere at 37 °C. The cell line S3, an oxaliplatin-resistant subline of TSGH [14], was maintained under conditions similar to those used for TSGH, except for a higher concentration of fetal bovine serum (10%) and addition of oxaliplatin (2 μg/mL). For all subsequent assays, resistant cells were cultured for at least 3 days without oxaliplatin in the media to avoid drug effect in influencing the result.

2.3. Microarray analysis

Total RNA was isolated from the TSGH and S3 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for microarray analysis. Labeled cDNA was hybridized to the Affymetrix GeneChip Test 3 Array to verify quality prior to hybridize to the Affymetrix Human Genome U133A Array. Reagents for hybridization, washing, and staining followed previous methods and the manufacturer's instructions [15]. The CEL files were transformed into intensity information using the RMA normalization of GeneSpring, and fold change was used to select differentially expressed genes.

2.4. Cell viability assay

Cells in logarithmic growth phase were cultured at a density of 1×10^4 cells/mL/well in a 24-well plate. The cells were exposed to various concentrations of the test drugs for the indicated times. At the end of the incubation period, cells were fixed and stained with 50% ethanol containing 0.5% methylene blue for 30 min. The plates were washed 5 times with water and allowed to air dry. The resulting colored residue was dissolved in 1% *N*-lauroyl-sarcosine, and optical density was read at 570 nm using a microplate reader. The IC_{50} value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with control growth. Each point represents the average of at least 2 independent experiments run in triplicate.

2.5. Interleukin-6 (IL-6) analysis

Culture medium was collected at the indicated times and clarified by centrifugation at 3000 × *g*. The IL-6 levels of the cell culture supernatants were determined using the commercially available IL-6 DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.6. Surface marker staining

Detection of cell surface antigen expression was performed as described previously [16]. Briefly, cells were harvested by treatment with 2 mM EGTA/PBS at 37 °C for 5 min. After washing with PBS, cells were suspended in 1× FACS staining buffer (1% FBS, 0.05% NaN_3 in PBS) containing IL-6 receptor (IL-6R) gp-80 and gp-130 monoclonal antibody (Life Technologies, Carlsbad, CA, USA) or matched isotype control (IgG_1 and IgG_{2a} for gp-80 and gp-130, respectively), then incubated at 4 °C for 30 min in the dark. Cells were then washed with 1× FACS staining buffer and resuspended in 1% paraformaldehyde/PBS. Flow cytometry was performed using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

2.7. Western blot analysis

Cells were initially seeded at a density of 1×10^6 in 100-mm^2 dishes. Adherent cells were collected and lysed with CelLytic™ M