Recent studies of 5-fluorouracil resistance in pancreatic cancer

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Abstract

Resistance to 5-fluorouracil (5-FU), an important anti-cancer drug, is a serious challenge in the treatment of pancreatic cancer. Equilibrative nucleoside transporter 1 and multidrug-resistance protein (MRP) 5 and MRP8, rather than P-glycoprotein, play important roles in 5-FU transport. Thymidylate synthase, dihydrofolate reductase, methylenetetrahydrofolate reductase and thymidine phosphorylase are four key enzymes involved in 5-FU metabolism. Other metabolic enzymes, including uridine monophosphate synthetase, also contribute to chemoresistance. Intracellular signaling pathways are an integrated network, and nuclear factor kappa-light-chain-enhancer of activated B cells, AKT and extracellular signal-regulated kinases are signaling pathways that are particularly relevant to 5-FU resistance. In addition, recent reports indicate that STAT-3 is a crucial survival protein. Proteomic assays provide a powerful tool for identifying target proteins and understanding the role of microRNAs and stromal factors to facilitate the development of strategies to combat 5-FU resistance.

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Key words: 5-fluorouracil; Resistance; Transporters; Metabolic enzyme; Signaling pathway; Stromal factors; MicroRNA; Proteomic investigation

Core tip: 5-fluorouracil (5-FU) is one of the most important drugs for human pancreatic cancer. Although recent studies have questioned the effectiveness of 5-FU against pancreatic cancer, it remains a good choice for pancreatic cancer. Our paper discusses recent studies that provide novel insights into 5-FU chemotherapy in pancreatic cancer.

INTRODUCTION

Pancreatic cancer is one of the most fatal types of cancer worldwide, accounting for 3% of new cancer cases and 6% of all cancer-related deaths in the United States[1]. The annual death rate for pancreatic cancer patients has remained stable over the past 10 years, and approximately 4% of patients survive for 5 years after diagnosis[2]. 5-fluorouracil (5-FU), a widely accepted anti-cancer drug, was first introduced in 1957[3]. As a pyrimidine analog, 5-FU exerts its anticancer effects through the inhibition of thymidylate synthase (TS) and the incorporation of its metabolites into RNA and DNA[4,5]. Despite initial doubts...
concerning the efficacy of 5-FU, numerous studies have since demonstrated a valuable role for 5-FU in combined treatment protocols compared with single gemcitabine chemotherapy\cite{8,20}. However, 5-FU chemoresistance, which may result from deficient drug uptake, alterations of targets, activation of DNA repair pathways, resistance to apoptosis and the tumor microenvironment, and other serious problems have been reported\cite{8}. In this review, we will discuss recent studies that provide novel insights into the mechanisms of 5-FU resistance.

TRANSPORT MECHANISMS

5-FU targets intracellular enzymes, and thus, the efficiency of 5-FU treatment depends on transport systems. However, there is little information regarding the role of transporters in mediating 5-FU resistance in pancreatic cancer. Nucleoside transporter systems, including human equilibrative nucleoside transporters (hENTs) and concentrative nucleoside transporters (hCNTs), particularly hENT1, play important roles in the cellular uptake and supply of nucleosides and nucleoside analogues. Tsujie et al\cite{14-16} reported that high expression of hENT1 mRNA led to low sensitivity to 5-FU in pancreatic cancer, which suggests that hENT1 plays an important role in 5-FU resistance and that hENT1 mRNA levels might be a useful marker to predict 5-FU sensitivity in pancreatic cancer. Furthermore, Gao et al\cite{10} observed that inhibition of hENT1 by dipyridamole (DP) could increase the intracellular concentration of 5-FU, thereby enhancing cytotoxicity in human pancreatic cancer cell lines. High expression of hENT1 may preferentially facilitate the uptake of nucleosides relative to 5-FU. Alternatively, hENT1 may provide a bilateral channel for 5-FU, whereas other transporters actively pump 5-FU into the cell. For example, 5-FU is a substrate of the human organic anion transporter 2 (hOat2, SLC22A7) but not hCNT1\cite{12}. Members of the ATP-binding cassette (ABC) transporter superfamily facilitate drug resistance via their role as efflux pumps. Interestingly, P-glycoprotein (P-gp, ABCB1), which is encoded by the multidrug resistance 1 gene (MDR1) and is the most common drug resistance ABC transporter, is not involved in 5-FU resistance\cite{13}, but the expression of multidrug-resistance protein 5 (MRP5, ABCG5)\cite{14-16} and MRP8 (ABCC11)\cite{17} is correlated with cellular 5-FU sensitivity.

The role of breast cancer resistance protein (BCRP, ABCG2) remains controversial. ABCG2 can transport the nucleotide CdAMP, similar to several other ATP-binding cassette transporters of the ABCC (multidrug resistance protein) family, and the nucleoside cladribine. In addition, the expression of ABCG2, a target gene of MSX2, correlates with chemoresistance in pancreatic cancer\cite{18,19}.

METABOLIC ENZYMES

Previous studies focused primarily on genes involved in 5-FU metabolism. Four intracellular enzymes are considered key determinants in controlling 5-FU sensitivity or resistance: thymidylate synthase (TS, TYMS), dihydropyrimidine dehydrogenase (DPD), methylenetetrahydrofolate reductase (MTHFR) and thymidine phosphorylase (TP)\cite{24}. The majority of studies have focused on gene polymorphisms or expression, and few have examined pancreatic cancer.

5-fluorodeoxyuridine monophosphate (5-FdUMP), the metabolite of 5-FU, directly binds to TS and inhibits its activity, catalyzing the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using 5,10-methylene tetrahydrofolate (CH2THF) as the methyl donor\cite{19}. Immunohistochemical analysis of paraffin-embedded tissues from 212 patients with pancreatic head or periampullary cancer following 5-FU-based adjuvant treatment revealed a significantly increased median survival of patients with low intratumoral TS expression compared with those patients with high TS expression\cite{20}. Furthermore, high TS expression was an independent predictor of poor prognosis. The TS gene can be classified into two different “alleles” based on the expression of a 28-bp variable number tandem repeat (VNTR) in the 5’ untranslated region (UTR) of TS: either two repeats (2R) or three repeats (3R) (with three common genotypes, 2R/2R, 2R/3R and 3R/3R)\cite{22}. In addition, the 3R allele can be subclassified according to the presence of a single nucleotide polymorphism (SNP) replacing a cytosine with a guanine (G/C) in 3R (3G or 3G)\cite{21}. An analysis of a panel of seven pancreatic cancer cell lines revealed that cells of the 3R/3R genotype, which express high levels of the TS protein, exhibit lower sensitivity to 5-FU compared with cells of the 2R/2R or 2R/3R or 3R/3R genotype\cite{21}. In a clinical study of patients with metastatic gastrointestinal cancer, Cui et al\cite{23} observed that patients with the 2R/3R genotype may be more sensitive to chemotherapeutic regimens, including 5-FU, than those with 3R/3R. By contrast, Hur et al\cite{23} observed no significant difference in the tumor responses of 3R/3R and 2R/3R patients. In a meta-analysis of 20 studies, Wang et al\cite{24} observed a significant increase in the overall survival of rectal cancer patients exhibiting the TS 3R/3R genotype. Taken together, these data highlight the need for further studies investigating the role of TS polymorphisms in 5-FU resistance.

TS, FdUMP and CH2FH4 form an inactive ternary complex that is stabilized by high CH2FH4 levels. MTHFR, a key regulatory enzyme involved in intracellular folate metabolism, converts CH2FH4 to 5-methyltetrahydrofolate (CH3FH4) and reduces 5-FU efficacy. The two most common polymorphisms linked to altered enzymatic activity are C677T and A1298C. In preclinical studies, the C677T mutation was associated with increased chemosensitivity of colon and breast cancers to 5-FU\cite{28}, and mutated A1298C variants exhibited enhanced 5-FU efficacy\cite{29}. Clinical studies performed by Delgado-Plasencia et al\cite{30} demonstrated that colorectal cancer (CRC) patients expressing variant T genotypes (CT or TT) at the C677T
polymorphism exhibited a higher survival rate after chemotherapy than the homozygote CC variant. No significant associations between the MTHFR c.1298 genotypes or MTHFR diploides and survival were observed[31].

More than 80% of administered 5-FU is catabolized by DPD in the liver. Thus, patients with DPD enzyme deficiency are at risk for developing serious 5-FU toxicity. Previous studies have demonstrated an association between DPD expression and patient survival. Immunohistochemical analysis of DPD expression in 176 patients with upper tract urothelial carcinoma (UTUC) revealed no significant association between DPD levels and patient prognosis. However, significantly higher levels of cell growth inhibition and a higher IC50 value for 5-FU were observed in UMUC-3 cells following targeted silencing of DPD by siRNA compared with controls[32]. Ciaparrone et al[33] demonstrated that CRC patients receiving adjuvant, systemic 5-FU and exhibiting high DPD expression had significantly shorter disease-free survival and overall survival compared with patients with low DPD expression. An analysis of 15 human pancreatic cancer cell lines and two 5-FU-resistant sub-lines revealed a significant correlation between 5-FU IC50 values and the expression of TS × DPD (quantitative analyses of mRNA expression levels), suggesting that pancreatic cancer cells with high TS and/or DPD levels are more resistant to 5-FU[34].

The first step of activation of 5-FU in tumor tissues involves the conversion of 5-FU to fluorodeoxyuridine by TP. TP, also referred to as platelet-derived endothelial cell growth factor, is an angiogenic factor that promotes angiogenesis in vivo and stimulates the in vitro growth of a variety of endothelial cells. The role of TP in the clinical response to fluoropyrimidine-based chemotherapy is complex. In a clinical study involving 35 patients with newly diagnosed, locally advanced pancreatic cancer who received radiotherapy with capcitabine, which is metabolized to 5-FU by TP, Saif et al[35] revealed that a lower TP/DPD mRNA ratio was significantly associated with higher overall survival. Miyake et al[36] also observed this association in a cohort of 25 pancreatic cancer patients following immunohistochemical analysis of the TP/DPD ratio in their surgical specimens.

Furthermore, Griffith et al[37] observed differential expression of uridine monophosphate synthetase (UMPS) isoforms in the MIP101 and MIP/5-FU CRC cell lines and demonstrated that a low UMP5 A/B isoform ratio, rather than the abundance of UMP5s mRNA, might be predictive of 5-FU resistance.

Taken together, these studies indicate that intracellular nucleoside metabolic enzymes are promising candidates as mediators of 5-FU resistance.

**ROLE OF GENES INVOLVED IN CELL CYCLE REGULATION, PROLIFERATION, REPAIR AND APOPTOSIS**

DNA and/or RNA damage caused by 5-FU leads to the activation of DNA repair systems or apoptosis. Thus, the alteration of genes involved in cell cycle regulation, proliferation, repair and apoptosis plays an important role in 5-FU resistance.

To investigate genes involved in 5-FU resistance, Wang et al[38] performed gene expression analysis using HG-U133A arrays in five breast cancer cell lines, including the 5-FU resistant cell lines MCF-7FU3, MCF-7RES and T47DRES and their drug-sensitive parental counterparts, MCF-7WT and T47DWT. Significant down-regulation of key genes involved in 5-FU activation was observed in 5-FU resistant cells, including TK, UMPK and OPRT. Furthermore, overexpression of genes involved in cell cycle regulation, proliferation, repair and apoptosis, including T5, c-YE3, NF-E2, p63 and c-Flip, was detected in the resistant cell lines. Cotransfection of NF-kB p50 and p65 cDNA induced 5-FU resistance in MCF-7 cells and reduced the expression of genes governing the G1-S and S-phase transitions. Cotransfection of NF-kB p50 and p65 cDNA induced 5-FU resistance in MCF-7 cells. Both NF-kB- and 5-FU-induced resistant cell lines exhibited reduced expression of genes governing G1-S and S-phase transitions. The expression of genes involved in DNA replication was also down-regulated in resistant cell lines. These findings were highly consistent with the slower growth rate, higher proportion of G1 cells and lower proportion of S-phase cells in the resistant cell lines. This phenotype may protect resistant cells from cell death induced by the incorporation of 5-FU into DNA chains by allowing time to repair 5-FU-induced damage[39].

Ischenko et al[40] tested the hypothesis that inhibition of Src tyrosine kinase could augment the chemosensitivity of the drug-resistant human pancreatic cancer cell lines AsPC-5-FU RES and L3.6pl-FU RES to 5-FU. The authors observed the following: (1) inhibition of Src tyrosine kinase activity by PP2 enhances 5-FU-induced cytotoxicity and induces apoptosis in 5-FU-resistant cells following 5-FU treatment; (2) Src specifically regulates 5-FU chemosensitivity in both the parental and chemotherapy-resistant cell lines; (3) overexpression of TS in chemotherapy-resistant cell lines is suppressed by PP2; and (4) 5-FU-induced EGFR-AKT pathway activation is affected by PP2 in chemotherapy-resistant cell lines; (5) overexpression of genes involved in 5-FU activation was observed in 5-FU resistant cells, including TS, 5-FU-induced resistant cell lines exhibited reduced expression of key genes governing the G1-S and S-phase transitions. The expression of genes involved in DNA replication was also down-regulated in resistant cell lines. These findings were highly consistent with the slower growth rate, higher proportion of G1 cells and lower proportion of S-phase cells in the resistant cell lines. This phenotype may protect resistant cells from cell death induced by the incorporation of 5-FU into DNA chains by allowing time to repair 5-FU-induced damage[39].

Taken together, these studies indicate that 5-FU resistance may be reversed by PP2, a Src tyrosine kinase inhibitor, via the EGFR-Akt pathway, by overcoming TS regulation. Zhao et al[41] also observed that pERK expression levels were noticeably increased in 5-FU-resistant SW1990/FU cells compared with their parental cell line. Treatment of SW1990/FU cells with the ERK inhibitor PD98059 sensitized cells to 5-FU by activating caspase-8 and reducing phospho-Bcl-2. Yoon et al[42] also reported that the AKT and ERK1/2 signaling pathways were activated in the 5-FU-resistant intradepathic cholangiocarcinoma cell line SCKR. Bcl-2 expression was also elevated in these cells, and the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 was capable of altering this phenotype.
Can et al\[43\] demonstrated the importance of a Ca\(^{2+}\)-calmodulin (CaM)-p53 axis in 5-FU-induced extrinsic apoptosis. Inhibition of this pathway using a Ca\(^{2+}\)-chelator or inhibitors of CaM abrogated the ability of 5-FU to activate caspase-8 and inhibited subsequent cell death. Furthermore, both TS inhibition and misincorporation of 5-FU metabolites into RNA result in p53 stabilization, and p53 may be involved in downstream signaling pathways in response to 5-FU\[45\].

Dictore et al\[49\] reported that aberrant constitutive activation of STAT3 protein is frequently detected in pancreatic adenocarcinoma, and type I interferons (IFNs), especially IFN-\(\alpha\), activated the JAK-2/STAT-3 pathway. Dictore et al\[49\] also reported the therapeutic role of peroxisome proliferator-activated receptor \(\gamma\) (PPAR-\(\gamma\)) in combination with other drugs (IFNs, gemcitabine and COX-2 inhibitors), highlighting molecular interactions and signaling pathways involved in pancreatic cancer cells, including Ras/Raf/MAPK pathway, Akt/PKB signaling, and Erk-1/2 pathway. Viale et al\[46\] treated human pancreatic cell line BxPC-3 with combination of recombinant IFN-\(\beta\) and PPAR-\(\gamma\) agonist troglitazone, and found a synergistic growth inhibition by MTT assay. Western blot analysis showed that IFN-\(\beta\)-induced activation of STAT3, MAPK, and Akt could be counteracted by TGZ-induced inactivation of STAT-3. The combination also decreased anti-autophagic bcl-2/bclcin-1 complex formation due to inactivation of the Akt-mTOR-dependent pathway. Spitzner et al\[46\] recently demonstrated that STAT-3 inhibition sensitizes colorectal cancer to 5-FU–based chemoradiotherapy (CT/RT) both in vitro and in vivo. Inhibition of STAT3 by RNAi-mediated silencing in both SW480 and SW837 cell lines exposed to 3 \(\mu\)mol/L of 5-FU and irradiation, and a subcutaneous xenograft model led to profound CT/RT sensitization. The inhibitory effect of STAT-3 in pancreatic cancer is worth expecting.

Cell signaling networks encompass numerous complicated pathways that involve significant crosstalk. To describe the main molecular mechanisms of 5-FU chemoresistance, we created an illustration based on many related studies; further studies are necessary to elucidate the roles of these networks (Figure 1).

### CONTRIBUTION OF STROMAL FACTORS TO DRUG RESISTANCE

Pancreatic cancer cells are typically surrounded by dense stroma. Stromal factors contribute significantly to the tumor microenvironment, but the role of the cancer microenvironment in 5-FU chemoresistance is just beginning to be explored. Sato et al\[47\] tested the sensitivity of MiaPaCa-2 and AsPC-1 cells to 5-FU following pre-incubation with recombinant annexin II (rANX II). In MiaPaCa-2 cells, treatment with rANX II led to the suppression of caspase-3 activation and increased Bcl-2/Bax ratio. Pre-incubation of cells with rANX II increased 5-FU resistance. Chen et al\[48\] demonstrated that the expression of focal adhesion kinase (FAK) related to 5-FU chemosensitivity involves an Akt/NF-kappaB signaling pathway in human CRC cells. Suppression of FAK expression significantly decreased 5-FU resistance and markedly increased the apoptosis of multicellular spheroid culture cells. Thus, 5-FU chemoresistance also requires the FAK/Akt/NF-kB survival signaling pathway. Expression of the obesity hormone leptin\[49\], stromal cell-derived factor-1\(\alpha\) (SDF-1\(\alpha\))/CXCR4 cross-talk\[50\], and \(\beta\)-integrin expression\[51\] have also been associated with 5-FU resistance in colon cancer cells. In addition to these classical signaling pathways, a new membrane receptor, calcium sensing receptor (CaSR), has also been shown to regulate drug resistance. Activation of CaSR by extracellular Ca\(^{2+}\) or its agonists enhanced the sensitivity of human colon carcinoma cells to 5-FU and down-regulated TS expression and the anti-apoptotic protein survivin\[52\]. Furthermore, the tumor-suppressive function of vitamin D in human colon carcinoma cells requires functional CaSR and promotes a cytotoxic response to 5-FU in a CaSR-dependent manner by suppressing the expression of TS and survivin\[53\]. Recent increased interest in pancreatic stellate cells should provide novel findings related to 5-FU resistance and the tumor microenvironment.

### MICRORNAS AND 5-FU RESISTANCE

MicroRNAs (miRNAs) are small, 19-25 nucleotide (nt), non-coding RNAs that function as post-transcriptional regulators capable of blocking the translation of mRNAs into protein and/or promoting the degradation of target mRNAs. Kurokawa et al\[54\] profiled the expression of miRNAs in DLD-1/R and KM12C/R cells, two 5-FU-resistant colon cancer sub-lines derived from the DLD-1 and KM12C cell lines, using Agilent human miRNA microarrays (G4471A) that included 723 human and 76 human viral miRNAs from the Sanger miRBase release 10.1. The authors identified the specific up-regulation of eight miRNAs in DLD-1/R and KM12C/R cells, in particular, miR-19b and miR-21. Subsequent miRNA: mRNA immunoprecipitation (RIP)-Chip analysis demonstrated that 66 mRNAs were recruited following the transfection of miR-19b into DLD-1 and DLD-1/R cells, including SFPQ (splicing factor proline and glutamate-rich), which has been linked to cell cycle function. SFPQ functions at different cell cycle stages to maintain sister chromatid interactions\[55\], and depletion of this gene has been shown to cause abnormal cell accumulation in the S phase of the cell cycle\[56\]. Similarly, Rossi et al\[57\] observed up-regulation of miR-19a (a paralog of miR-19b) and miR-21 in HT29 and HCT-119 colon cancer cells in response to 5-FU exposure. The majority of miR-21 targets are tumor suppressors, including PTEN\[58,59\], PDCD4\[60\] and Bel-2\[60\]. By performing in silico analysis coupled to experimental validation, Boni et al\[60\] determined that miR-192 and miR-215 target TYMS expression in CRC cell lines; however, down-regulation of TYMS by miR-192/215 did not sensitize CRC cell lines to 5-FU treatment. Based on these results, the au-
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Figure 1 Pancreatic cancer cell survival pathways in 5-fluorouracil resistance. DNA and/or RNA damage caused by 5-fluorouracil (5-FU) leads to the activation of DNA repair systems or the apoptosis cascade. Several cell survival pathways, including the epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) pathway, Akt/mechanistic target of rapamycin (mTOR) pathway, STAT3 dependent pathway, phosphatidylinositol 3-kinase (PI3K)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and Wnt/GSK3β/β-catenin pathway, are involved in 5-FU resistance in pancreatic cancer.

Proteomic investigation of 5-FU resistance
Proteomics is a powerful tool for detecting and identifying drug resistance-related proteins. Two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry (MS), such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), is now a commonly employed method. A significant number of studies have investigated the mechanisms under-
ing gemcitabine resistance in pancreatic cancer cells[82-84], and similar studies related to 5-FU resistance are now underway. Yoshida et al[85] identified 40 differentially expressed protein spots between TS-1-resistant cells, PK45p and KLM-1, and TS-1-sensitive cells, Panc-1, BxPC-3, MiaPaCa-2 and PK59, using 2-DE and LC-MS/MS. TS-1 is a mixture of 5-FU and tegafur (FT), a metabolically activated prodrug of 5-FU. Among the 40 differentially expressed proteins, 29 were up-regulated, including hypoxia up-regulated protein 1 (oxygen regulated protein, ORP150) and annexin A1. Kimura et al[86] identified two ribosomal proteins, L15 and L37, by proteomic analysis of DLD-1 and DLD-1/5-FU cells by 2-DE and MALDI-TOF/TOF-MS/MS. Tan et al[87] identified 102 unique proteins, including p16, Maspin, PRDX6, PSMB7, MYL6, PHB and HSP27, in the altered hepatocellular carcinoma (HCC) cell line SMMC-7721/5-FU compared with parent cells. Furthermore, down-regulation of PRDX6 and PSMB7 enhanced 5-FU sensitivity in SMMC-7721/5-FU cells. Isobaric tags for relative and absolute quantitation (iTRAQ) is a non-gel-based technique used to quantify proteins from different samples in a single experiment followed by LC-MS/MS[88]. Using this technique, Tong et al[89] identified 52 proteins that were differentially expressed in the HCC cell line BEL7402/5-FU compared with its 5-FU-sensitive counterpart, BEL7402. Of these 52 differentially expressed proteins, 26 were increased in BEL7402/5-FU, notably annexin A3 (ANX3), one of the least-studied members of the annexin family. Importantly, suppression of ANX3 led to the enhancement of 5-FU sensitivity in BEL7402/5-FU cells. Although these experiments are associated with inherent technical variability, proteomic studies provide new targets for investigating novel mechanisms of 5-FU resistance.

CONCLUSION

More than 60 years after its development, 5-FU continues to be an important anticancer drug. However, more significant studies are required to understand the mechanisms underlying 5-FU resistance in pancreatic cancer. Screening at the genomic and proteomic levels has provided an abundance of candidate targets, and summarizing these studies and applying this knowledge for the development of successful 5-FU-based treatment strategies are essential. With comprehensive databases, the analysis of signaling networks, protein-protein interactions and intracellular-extracellular crosstalk is now possible. Multi-drug resistance studies have increased interest in drug-directed research. The study of chemo-resistance mechanisms is likely to promote the application of novel, successful combination chemotherapy protocols for improved outcomes for cancer patients.

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