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Establishment and characterization of the gemcitabine-resistant human gallbladder cancer cell line NOZ GemR

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Background: Patients with gallbladder cancer (GBC) generally receive gemcitabine as the standard treatment; however, its efficacy is often limited owing to the development of resistance.
Methods: To identify the mechanisms underlying gemcitabine resistance in GBC, a gemcitabine-resistant GBC cell line (NOZ GemR) was established by exposing the parental NOZ cell line to increasing concentrations of gemcitabine. Morphological changes, growth rates, and migratory and invasive capabilities were evaluated. Protein expression was detected using western blotting.
Results: The results demonstrated that the IC_{50} of NOZ and NOZ GemR was 0.011 and 4.464 μM, respectively, and that the resistance index ratio was 405.8. In comparison, NOZ GemR cells grew slower and had significantly lower migration and invasion abilities than NOZ cells. There were altered levels of epithelial-mesenchymal transformation markers in NOZ GemR cells, as well as increased levels of the Akt/mTOR pathway protein.
Conclusion: The NOZ GemR cell line could be used as an effective in vitro model to improve our understanding of gemcitabine resistance in GBC.

Keywords: chemotherapy, drug resistance, gallbladder cancer, gemcitabine

Introduction

Gallbladder cancer (GBC) is the most common type of malignant tumor of the biliary tract with a 5-year survival rate of less than 5%[1]. Due to the aggressive nature of the tumor, its late presentation, complex anatomic position, and advanced stage at diagnosis, the prognosis for GBC is poor[2,3]. In the present day, there are no effective methods for GBC prevention or treatment. Only a small proportion (approximately 10%) of patients with early-stage GBC are suitable candidates for curative treatment that primarily involves surgery as the sole curative option[4]. Palliative chemotherapy is used to treat unresectable, locally advanced, and metastatic diseases. As a result, chemotherapy has become crucial in the clinical management of patients with GBC[5]. Gemcitabine-based chemotherapy is the first-line treatment for patients with advanced GBC. Nevertheless, the effectiveness of gemcitabine in GBC has diminished due to the rise in gemcitabine resistance (GemR)[6,7].

HIGHLIGHTS

- We have successfully established a human gemcitabine-resistant gallbladder cancer cell line.
- Proliferation, migration, and invasion of NOZ GemR cells are decreased.
- Vimentin was downregulated in the gemcitabine-resistant cell line.
- NOZ GemR cells exhibit elevated levels of Akt/mTOR signaling proteins.

Gemcitabine, a nucleoside analog of deoxycytidine, functions by directly disrupting DNA synthesis, inhibiting ribonucleotide reductase, and impeding cell cycle progression during the transition from the G1 to S phase[8]. GemR may occur due to cellular alterations such as increased DNA damage repair capacity, cell cycle dysfunction, accelerated drug efflux, activation of cell stem cells, and epithelial-mesenchymal transformation (EMT). GemR is also linked to various molecular factors, including dysregulation of transcription factors, genes related to oxidative stress, miRNAs, long noncoding RNAs, and circular RNA[9–12]. Despite extensive investigation, the underlying causes of GemR in GBC remain unclear[13,14]. Hence, it is crucial to establish gemcitabine-resistant GBC cell lines to delve deeper into the mechanisms behind GemR and devise a successful plan to combat drug resistance and enhance the effectiveness of treatment.

In this study, we established a GBC cell line, NOZ GemR, which is resistant to gemcitabine, and conducted biological and

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molecular analyses to characterize it. Our data offers a valuable framework for investigating novel resistance mechanisms and alternative therapeutic approaches for GBC.

Materials and methods

Cell line and cell culture

NOZ cells were provided by Genechem (Genechem Incorporation), and short tandem repeat analysis was performed to verify the authenticity of the cells as previously described[15]. NOZ cells were cultured in RPMI-1640 medium (Servicebio) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics (Servicebio). The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Establishment of the GEM-resistant cell line

To establish gemcitabine-resistant NOZ GemR cells, a stepwise increase in gemcitabine concentration (Sellect) was administered to NOZ cells over time. NOZ cells were cultured in RPMI-1640 containing 0.005 μM gemcitabine at 37°C for 48 h, after which the culture medium and dead cells were replaced with a fresh drug-free medium. Following the above conditions, the remaining cells were cultured until their growth reached logarithmic phase. The cells were passaged and re-cultured in a medium containing 0.005 μM gemcitabine until they stabilized. Following medium replacement, a culture medium containing 0.01 μM gemcitabine was prepared and cultured with cycle progress, according to a two-fold increase in the drug concentration. A stable gemcitabine-resistant cell line, designated as NOZ GemR, was successfully established after 12 months. Finally, the cells were stably cultured in medium containing 10 μM gemcitabine. The resistance index was calculated as the ratio of the IC_{50} values of the parental and resistant cells. Drug-resistant GBC cells were grown in drug-free medium for 2 weeks before being harvested, frozen in liquid nitrogen, and stored at – 80°C until analysis.

Morphologic appearance of NOZ cells and NOZ GemR cells

NOZ and NOZ GemR cells were cultured separately in 25 cm^2 culture flasks under 5% CO_2 and 37°C. Morphological examination of NOZ and NOZ GemR cells was conducted under an optical microscope when they entered the logarithmic phase.

Cell proliferation and colony formation assay

Cells were seeded into 96-well plates at a density of 3.5×10^3 per well in 100 μl of cell culture medium and cultured for the indicated time periods. The Cell Counting Kit-8 (CCK-8; Bimake) was used to measure cell proliferation. We performed a cellular colony assay using 1000 cells plated in 6-well plates and cultured them for 2 weeks. The cells were fixed in 70% ethanol and stained with crystal violet (in 0.5% ethanol).

Transwell migration and invasion assays

A total of 2×10^4 and 3×10^4 cells (for migration and invasion, respectively) were seeded into the upper chamber of 8 μm-pore transwell inserts (Corning) in serum-free RPMI-1640 and allowed to migrate and invade for 24 h toward the lower chamber containing 600 μl of RPMI-1640 with 10% FBS. To conduct invasion assays, Transwell inserts were coated with 20 μg Matrigel (Corning). To detect cell migration, cells were stained with 4% paraformaldehyde and 0.1% crystal violet staining was used.

Western blot analysis

Total protein was extracted from the cells using RIPA lysis buffer containing protease inhibitors (Beyotime) and quantified using the Piece BCA Protein Assay Kit (Thermo Scientific). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred on to polyvinylidene fluoride membranes after extraction. Next, we blocked the membrane with 5% nonfat milk in 0.1% PBST and incubated the membrane with the indicated antibodies. The following primary antibodies were used: β-Actin (A5441, Sigma), Akt (#4685, CST), p-Akt (Ser473) (#4060, CST), p-p70 S6K (Thr389) (#9234, CST), p-mTOR (Ser2448) (#5536, CST), vimentin (#5741, CST), Slug (#9585, CST), and Snail (#3879, CST). Detection of the antigen-antibody complex on the membrane was achieved using enhanced chemiluminescence (Bio-Rad). Blots were quantified using Image J software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9. Unpaired Student's *t*-test was used to analyze the between-group effects of gemcitabine on NOZ and NOZ GemR cells. The growth of NOZ and NOZ GemR cells was assessed using repeated measures two-way analysis of variance (ANOVA) with group as the between factor and day as the within factor followed by a least significant difference (LSD). $P < 0.05$ was considered to indicated a statistically significant.

Results

Establishment and characterization of gemcitabine-resistant cancer cell line NOZ GemR

During the past 12 months, NOZ cells were exposed to progressively higher concentrations of gemcitabine to establish a stable gemcitabine-resistant cell line, NOZ GemR. The increase in gemcitabine concentration ranged from 0.001 to 25 μM in NOZ cells. The gemcitabine-resistant cell line NOZ GemR showed a higher IC_{50} than that the parental cell line (4.464 vs. 0.011 μM for NOZ GemR and NOZ, respectively, and $P < 0.001$), with a resistance index of 405.8 (Fig. 1A). After 3 months of passage in gemcitabine-free medium, the IC_{50} value of NOZ GemR cells remained unchanged. Cells stored at – 80°C for 4 months also showed similar stability. The morphology of the gemcitabine-resistant cells differed in size and shape. A notable feature was the elongation of most gemcitabine-resistant cells, with more filopodia and lamellipodia around the cell periphery (Fig. 1B).

NOZ GemR cells possess a decreased ability for proliferation, migration, and invasion

A significant difference in growth rate was observed between NOZ GemR and NOZ cells. In contrast to parental NOZ cells, gemcitabine-resistant cells showed a markedly reduced growth rate ($P < 0.01$), with a significantly longer growth time of 38.37 h for NOZ GemR cells as opposed to 24.85 h for parental NOZ cells (Fig. 2A). Additionally, NOZ GemR colony formation was

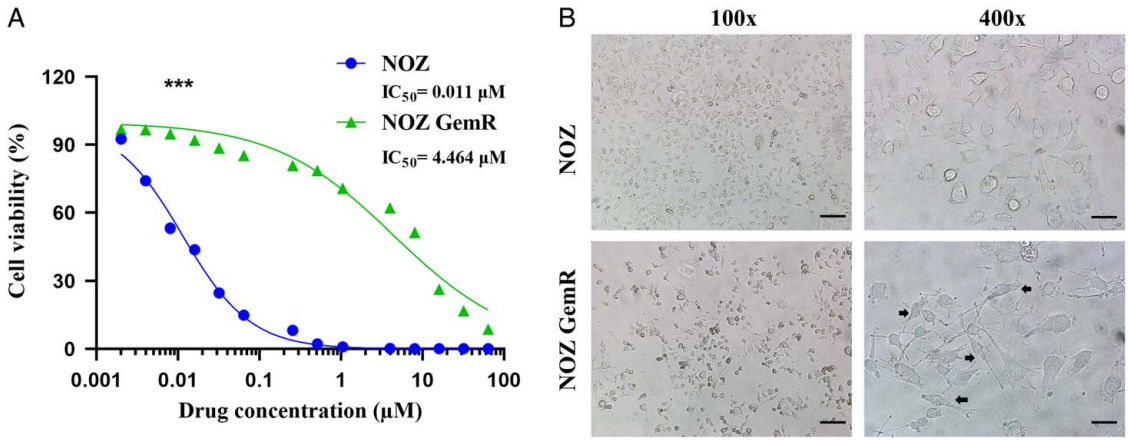

Figure 1. Characteristics of gemcitabine-resistant gallbladder cancer cells. A. Dose-response curves for NOZ and NOZ GemR cells following 72 h of treatment with the indicated doses of gemcitabine (***$P < 0.001$). B. Bright-field images showing the morphological characteristics of NOZ and NOZ GemR cells (100× magnification left and 400× magnification right), and fusiform-shaped cells are indicated by black arrowheads.

markedly reduced ($P < 0.01$) in an assay measuring colony formation (Fig. 2B). In the Transwell migration and Matrigel invasion assays (Fig. 3A), NOZ GemR cells exhibited significantly reduced migration and invasion abilities ($P < 0.01$).

NOZ GemR cells show altered protein levels of EMT markers and the Akt/mTOR signaling pathway

EMT protein markers were analyzed by western blotting. Slug and vimentin expressions were higher in NOZ GemR cells than in parental cells, whereas Snail expression was lower. However, N-cadherin and E-cadherin were not detected (Fig. 3B). As part of our investigation of GemR in NOZ GemR cells, we evaluated activation of the Akt/mTOR signaling pathway. Significant upregulation of p-Akt (Ser473), p-mTOR (Ser2448), and p-p70 S6K (Thr389) protein expression was observed in NOZ GemR cells, whereas total Akt expression was not altered (Fig. 3B).

Discussion

In this study, a gemcitabine-resistant NOZ GemR cell line was generated by exposing the corresponding parental NOZ cells to gradually increasing concentrations of gemcitabine over the course of a year. Characterization of the resistant subline NOZ GemR was performed by comparison with the parental NOZ cells. The IC_{50} value of NOZ GemR was 4.464 μM, which was far higher than those of the previously reported gemcitabine-resistant GBC cell lines, NOZ GemR and TGBC1 GemR, with IC_{50} values of was 0.203 and 0.891 μM, respectively[13]. Despite continuous culture in drug-free medium for 3 months and storage at – 80°C for 4 months, GemR remained stable.

As previously observed for other gemcitabine-resistant cells[8,16,17], the growth curve of NOZ GemR cells was constructed and it was determined that their growth rate was significantly lower than that of NOZ cells (Fig. 2). This may be because gemcitabine kills tumor cells by disrupting DNA synthesis. Cells in the proliferative phase with active DNA synthesis were inhibited earlier by intermittent treatment with gemcitabine during the establishment of the NOZ GemR. NOZ cells with slow growth rates and gemcitabine sensitivity are left behind to form gemcitabine-resistant cell lines, allowing DNA repair over a longer period of time[8,18].

In contrast to an earlier report by Vergara-Gómez *et al.*[13], the migratory capacity and invasive ability of NOZ GemR cells was significantly reduced in our study (Fig. 3A). This may be due to a higher resistance index (405.8 vs 32.8) or tumor heterogeneity[19,20]. Vimentin downregulation was observed in

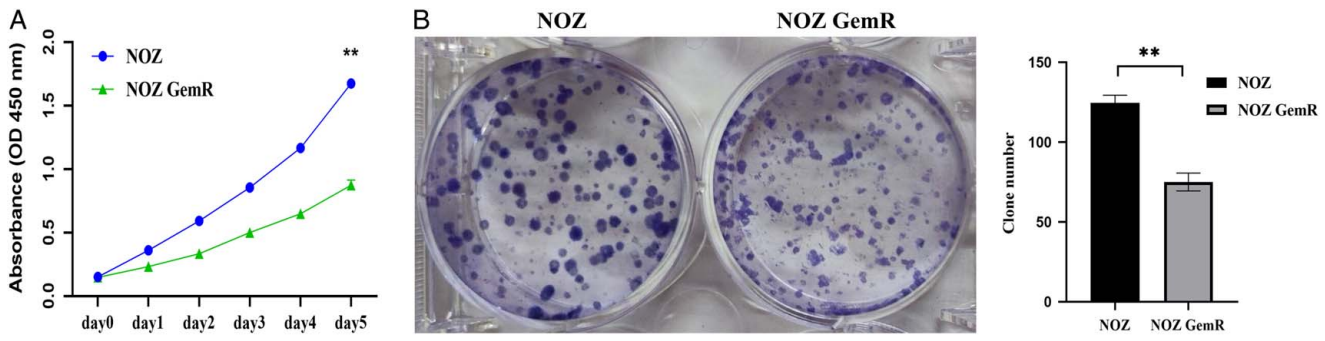

Figure 2. Inhibition of proliferation, colony formation of NOZ GemR cells. A. CCK-8 assays were used to detect cell growth upon NOZ and NOZ GemR cells (**$P < 0.01$). B. Colony formation assay was performed to examine the colony formation ability of gemcitabine-resistant gallbladder cancer cells (**$P < 0.01$).

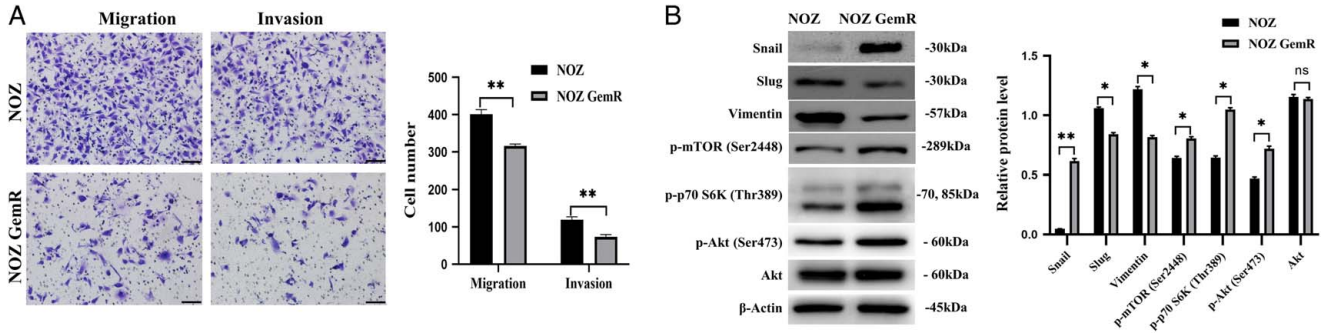

Figure 3. NOZ GemR cells exhibit changes in EMT marker expression and the Akt/mTOR signaling pathway. A. Transwell assay was performed to detect the migration and invasion of gemcitabine-resistant GBC cells (100× magnification) (**$P < 0.01$). B. The expression of β-Actin, Akt, p-Akt (Ser473), p-p70 S6K (Thr389), p-mTOR (Ser2448), vimentin, Slug and Snail in NOZ and NOZ GemR cells were examined by western blotting, each group was replicated three times (*$P < 0.05$, **$P < 0.01$).

the gemcitabine-resistant cell line NOZ GemR compared to that in the drug-sensitive control (Fig. 3B). The effects of gemcitabine can be seen in the reduction of vimentin expression and epithelial-mesenchymal transition in pancreatic cells[21]. In previous studies, vimentin expression was found to be downregulated in drug-resistant ovarian cancer cell lines[18,22,23], indicating a correlation between low vimentin expression and chemotherapy resistance. Huo *et al.*[18] revealed that vimentin silencing upregulated proteins involved in the exocytotic process and induced prolonged G2 phase arrest and the acquired stem cell-like phenotype, which contributes to drug resistance. Therefore, vimentin may be a potential therapeutic target for the treatment of GemR.

The expression of p-Akt (Ser473), p-mTOR (Ser2448), and p-p70 S6K (Thr389) proteins was significantly increased in NOZ GemR cells (Fig. 3B). Based on this study, it is possible that the Akt/mTOR signaling pathway plays an important role in the regulation of GemR in NOZ cells. It has been shown that Akt is a crucial upstream regulator of mTOR, and that activation of Akt may result in mTOR/p70 S6K activation. Through activation of the Akt /mTOR/p70 S6K axis, apoptosis can be suppressed, while protein synthesis and cell survival are promoted. Akt activation is involved in GemR in nonsmall cell lung cancer[24]. The fact that mTOR has been found to be a ubiquitous driver of therapy resistance indicates that mTOR inhibitors hold immense promise as a means of addressing cancer drug resistance[25,26]. There should be more research conducted on Akt/mTOR signaling and the potential for agents targeting Akt/mTOR to reverse GemR in vitro. There is a limitation to this study in that it is primarily descriptive. In order to understand the exact molecular mechanism behind GemR in GBC, transcriptomic and proteomic analyses are needed.

Conclusion

In this study, a gemcitabine-resistant NOZ GemR cell line was successfully established in this study. Therefore, this cell line may provide a useful model for studying the molecular mechanisms of GBC drug resistance and may lead to novel therapeutic strategies.

Ethical approval

The study has been approved by the Ethics Committee of Bengbu Medical College (Bengbu, China) (approval no. 37 on the 13 March 2023). It could be provided on request.

Consent

None. It was a preclinical study using commercial cell line. No patients were involved in this experiment. Thus, consent was not required for the current study.

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Author contribution

M.X.: conception or design of the work; B.J. and S.X.: performed experiments and data analyses; Z.M.: conceived and supervised the project, reviewed and edited the manuscript; M.X.: drafting the article; Z.M.: critical revision of the article. All authors reviewed the final version of the manuscript critically and gave the final approval.

Conflicts of interest disclosure

The authors have no conflicts of interest to declare.

Research registration unique identifying number (UIN)

None. It was a preclinical study using commercial cell line. No patients were involved in this experiment. Therefore, Research Registration was not required for this study.

Guarantor

Zhongran Man.

Data availability statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Provenance and peer review

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