Original Article
Correlation of β-tubulin III and ERCC1 mRNA expression to chemotherapy outcome of gastric cancer patients with malignant ascites

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Abstract: We sought to determine how intraperitoneal administration of docetaxel (DTX) or cisplatin (DDP) plus tegafur-gimeracil-oteracil potassium (S-1) related to the expression levels of two genes, TUBB3 and ERCC1, when given to gastric cancer patients with peritoneal carcinomatosis. DTX or DDP was administered intraperitoneally via an implanted catheter, whereas S-1 was orally administered. We developed a new response criterion through B ultrasound to evaluate treatment against malignant ascites. The primary endpoint for treatment effectiveness was the 1-year overall survival (OS) rate. The secondary endpoints were the response rate, efficacy against malignant ascites and treatment safety. These results were correlated to the expression levels of TUBB3 and ERCC1. Peritoneal carcinomatosis was synchronous and metachronous in 80% and 20% of patients, respectively. Patients who received selective intraperitoneal perfusion chemotherapy which took into account gene expression levels had a median survival time of 247.5 days. Clinical regression of ascites and related symptoms was observed in all patients. One patient experienced hematologic toxicity. Malignant ascites disappeared in one patient after treatment. Overall, the 1-year OS rate was 40%. Intraperitoneal DTX or DDP with oral S-1 was well tolerated, efficacious and safe in gastric cancer patients with peritoneal metastasis and correlated to gene expression levels.

Keywords: Gastric cancer, malignant ascites, class III beta-tubulin (TUBB3), excision repair cross-complementation group 1 (ERCC1), intraperitoneal chemotherapy

Introduction

Gastric cancer is the fourth most common cancer [1, 2], and is highly prevalent in many Asian countries, especially in China [3, 4]. For stage IV gastric cancer, peritoneal carcinomatosis may occur through dissemination into the peritoneal cavity [5]. Peritoneal disseminations are frequent, with about 53-60% of gastric cancer deaths evolving peritoneal carcinomatosis [6]. The response rate in gastric cancer patients with peritoneal metastases is very low and peritoneal carcinomatosis of gastric cancer generally has a poor prognosis with a median survival time of 1-3 months [6-8]. Systemic treatment is not generally successful, and so, the development of localized treatment strategies is important.

Since the 1980s, intraperitoneal chemotherapy has been used to treat metastatic ovarian cancer, and has demonstrated high clinical effectiveness [9]. Of potential application is the combined administration of tegafur-gimeracil-oteracil potassium (S-1) with intraperitoneal docetaxel (DTX) for patients with positive cytology in peritoneal lavage specimens or with macroscopically visible peritoneal metastasis [10]. From previous studies it has been found that a combination of DTX, carboplatin, and 5-fluorouracil has been useful in intraperitoneal chemotherapy [11].

Currently there is no standard regimen for the treatment of malignant ascites that arise from peritoneal carcinomatosis of advanced gastric cancer. As such, it is important to identify bio-

markers that can be used to predict patient response to chemotherapy. To some extent, variations in genes between patients can enhance or reduce the effectiveness of chemotherapy. Gene expression levels can therefore be used as prognostic and/or predictive markers for chemotherapy selection [12]. The excision repair cross-complementing group 1 (ERCC1) has been associated with resistance to cisplatin (DDP), and as such, may be a useful marker to predict treatment success [13-15].

From previous work, it has been established that the reversal of class III β-tubulin (TUBB3) expression is related to the clinical outcome for different cancers [16-19]. Despite this, the importance of TUBB3 in the treatment of malignant ascites of gastric cancer has not been studied in detail. Thus, TUBB3 status may provide important information in selecting options for the treatment of advanced gastric cancer with malignant ascites.

In this study we sought to examine the expression levels of TUBB3 and ERCC1 in malignant peritoneal effusion from carcinomatosis of advanced gastric cancer. We then used this information to determine whether gene expression levels afforded sensitivity to chemotherapy using DTX or DDP as model drugs when administered via intraperitoneal delivery. Specifically, we sought to determine how the effectiveness and safety of the chemotherapy related to mRNA expression in malignant ascites of gastric cancer.

Patients

Patients were enrolled between January 2013 and March 2014. The research was conducted by the Department of Oncology, the Third Affiliated Hospital, Soochow University, P. R. China. Peritoneal metastases were diagnosed by biopsy using laparotomy, laparoscopy, or by the cytologic examination of ascites. All patients used in the study were diagnosed with malignant ascites.

Three patients (60%) included in the study were male. The average age was 57.4 years (range 45-68 years). One patient had previously undergone surgery to treat primary gastric cancer with a curative intent, and this patient was synchronous with metastasis. All other patients in the study presented with metachronous metastasis. Gastric cancer was considered unresectable in the four other cases at the time of the diagnosis and no gastric surgery had been performed.

Eligibility criteria for enrollment into the trial included a minimum patient age of 18 and an Eastern Cooperative Oncology Group performance status of 0 to 2. Patients had adequate hematologic function (i.e., hemoglobin > 9.0 g/L, absolute neutrophil count > 1500/IL, and platelets > 100,000/IL), hepatic function (i.e., total bilirubin <2 times the upper limit of normal, alanine aminotransferase and aspartate aminotransferase levels <2.5 times the upper limit of normal), renal function (i.e., serum creatinine <1.5 mg/dL, creatinine clearance <50 mL/min), and normal electrocardiogram results.

Informed consent according to institutional guidelines was obtained from all patients. This study was approved by the ethics committees of Soochow University.

Effusion sampling and cell preparation

Following careful disinfection of the skin, ARROWg+ and blue central venous catheters (Arrow International Inc., Reading, PA, USA) were inserted into the peritoneal spaces. Specimens for extraction of cell-free RNA were collected in a sterile drainage bag containing heparin (10,000 U/L effusions) and were immediately transported on ice to the laboratory. The cells were prepared by centrifuging the effusions at 1000×g for 10 min, before the cells were assayed following the procedures given below.

Methods

Tumor ERCC1 and TUBB3 mRNA expression assay

Shanghai Yuanqi Biomedical Technology Limited (Shanghai, P. R. China) performed the experiments. The mRNA of tumor cells were separated, according to the genomic RNA extraction kit instructions, the RNA extraction ratio for OD260/OD280 was 1.8 to 2.0. Tumor ERCC1 and TUBB3 mRNA gene expression levels were determined (PCR fluorescence probe method) and the ABI 7300 quantitative fluorescence determination of tumor-associat-

Table 1. Patient characteristics, complications and survival results

Patient	Sex	Age	Synchronous/ metachronous	Chemo-agent	IP times	Complications	Reason of death	Survival (days)
F.H.	M	54	synchronous	docetaxel	6	hematologic toxicity	gastric cancer	367
F.S.	M	64	metachronous	cisplatin	6	No	gastric cancer	212
C.X.	F	56	synchronous	docetaxel	6	No	survival	454+
C.Z.	M	68	synchronous	cisplatin	6	bowel obstruction	gastric cancer	64
W.W.	F	45	synchronous	cisplatin	6	No	gastric cancer	247

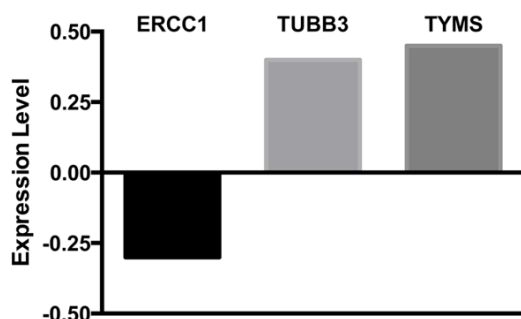"+" represents that the patient was still alive at the time of writing this report.

Figure 1. One patient with malignant ascites from peritoneal carcinomatosis of advanced gastric cancer, showing low-level expression of ERCC1, and high-level expression of TUBB3.
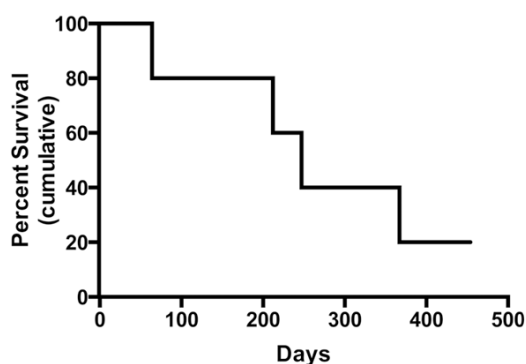

Figure 2. Overall survival of the five patients with peritoneal dissemination of gastric cancer. Mean survival time was 247.5 days, and the 1-year OS rate was 40%. Median follow-up time was 267 days.

ed amplification was completed by Shanghai Yuanqi Biomedical Technology Limited.

Treatment protocol

The expression levels of TUBB3 and ERCC1 were evaluated to inform the chemotherapy treatment selection for intraperitoneal delivery via the abdominal cavity to each patient. As

low-level expression of ERCC1 confers sensitivity to platinum-based chemotherapy, we selected DDP as the platinum drug. Likewise, as low-level expression of TUBB3 confers sensitivity to treatment with microtubule binding drugs, we choose DTX as the taxane for use in this study.

For cancers expressing low levels of TUBB3, DTX was administered intraperitoneally on day 1 at a fixed dose 60 mg/m^2 [10], or DDP was administered intraperitoneally on day 1 at a fixed dose of 75 mg/m^2, and S-1 was administered at a fixed dose of 80 mg/m^2/day for 14 consecutive days, followed by 7 days of rest [10].

To ensure that the drugs were fully available on the peritoneal surface, each patient was tilted at 15-min intervals after perfusion as follows: (1) Patient kept level, (2) Trendelenburg + left tilt, (3) Trendelenburg + right tilt, (4) Patient kept level, (5) Reverse Trendelenburg + left tilt, (6) Reverse Trendelenburg + right tilt.

All patients completed six cycles of chemotherapy unless they experienced unacceptable side effects from the treatment.

Evaluation of the disease

Malignant ascites are considered to be non-evaluable lesions. In this study, we developed a new response criteria for treatment against malignant ascites, which is similar to the modified WHO criteria of efficacy assessment in malignant tumors [20]. Before and after intraperitoneal perfusion chemotherapy, ultrasonic examination was performed to assess the clinical response after two cycles of treatment. Clinical efficacy was divided into three grades: complete response (CR), which is defined as no detection of effusion in the B ultrasonic or CT scan (all tumors have disappeared); partial

Table 2. Carcinoembryonic antigen level of abdomen effusion before hyperthermic intraperitoneal perfusion chemotherapy and after two cycles after intraperitoneal administration, and the results of abdomen effusion therapy

Patient	Abdomen effusion CEA before HIPC	Abdomen effusion CEA after two times perfusion later	Therapy result of effusion
F.H.	1000	1000	PR (decrease of ascite ≥50%)
F.S.	97	32	CR (disappear of ascite)
C.X.	96.2	16.69	PR
C.Z.	24.19	11	PR
W.W.	24.96	3.71	PR

response (PR), which is defined as B ultrasound examination above the bladder with longitudinal section that shows the ascites' depth was decreased by at least 50%; and, non-partial response (nPR), which is defined as an insufficient decrease in tumor size, or a decrease in the depth of the ascites of less than 50%, or an increase in the depth above the bladder with longitudinal section. The primary endpoint was the 1-year OS rate. Secondary endpoints were the response rate against malignant ascites and safety.

Statistical analysis

Outcome data were obtained from the patient's medical records and via patient interview. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL USA). Survival was calculated from the initial date of treatment to either the death of the patient or to the date of the most recent follow-up visit using the Kaplan-Meier method. Univariate analysis was performed using the log-rank test. All statistical analyses were two-sided tests, with a *p* score of less than 0.05 considered as giving a statistically significant difference.

Results

Patients were enrolled between January 2013 and March 2014. A total of five gastric cancer patients with malignant ascites were enrolled in this study, with all five showing positive peritoneal lavage cytology under local anesthesia. The clinical characteristics of the five patients are listed in **Table 1**. One patient who underwent gastrectomy presented with metachronous metastasis, while the other four patients presented with synchronous metastasis. The

patient group comprised three men and two women with a mean age of 57.4 years (range 45-68 years). All five patients were available for follow-up with the following survival times: 367, 212, 454[+], 64 and 247 days. Those patients who received intraperitoneal chemotherapy, which was selected based on gene expression levels, had a median survival time of 247 days. In total there were 30 cycles of chemotherapy given to the patients. Of all the patients treated, there were no deaths related to the procedure. The grade 3/4 grade toxic effects were neutropenia; leucopenia only occurred once in one patient, and one patient developed an intestinal occlusion during their last cycle of intraperitoneal chemotherapy.

Figure 1 gives an example of the gene expression results of one patient with malignant ascites from peritoneal carcinomatosis of advanced gastric cancer, showing the low-level expression of ERCC1 and high-level expression of TUBB3.

Figure 2 shows the overall survival time after the introduction of intraperitoneal perfusion chemotherapy for all patients enrolled in this study and **Table 2** shows the effect of peritoneal dissemination after therapy. Clinical regression of ascites and related symptoms was achieved in all patients. Malignant ascites disappeared in one patient after intraperitoneal perfusion chemotherapy as selected by gene expression. The results shown are for patients after two cycles of chemotherapy. For effusion, the mean carcinoembryonic antigen level decreased from a pre-chemotherapy value of 248.5 to a post-chemotherapy value of 212.7 (P<0.05). The mean carcinoembryonic antigen level in serum decreased from a pre-chemotherapy value of 209.4 to a post-chemotherapy value of 118.2 (P<0.05, **Table 2**). Overall survival at 6, 9, and 12 months was 80%, 40%, and 40%, respectively. Median follow-up time was 267 days (**Figure 3**). **Figure 3** shows the response evaluation before and after intraperitoneal chemotherapy perfusion as determined using B ultrasound.
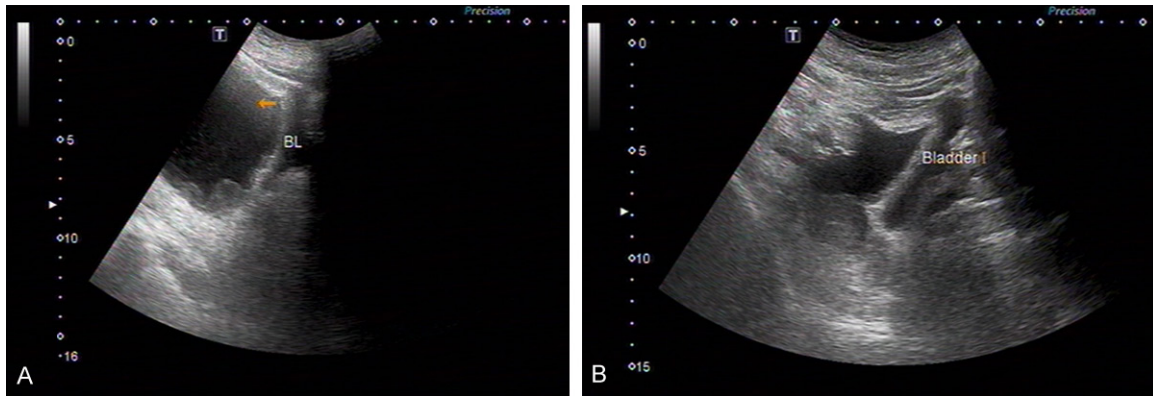
Figure 3. (A) An abdominal effusion before analysis of the gene expression levels and therapy as determined using B ultrasound (Yellow arrow: Abdomen effusion, BL: Bladder), and (B), abdominal effusion after two cycles of therapy as determined using B ultrasound (abdomen effusion located to the left of the bladder).

Discussion

Generally the prognosis for patients with peritoneal carcinomatosis (PC), which has a gastric origin is not good; peritoneal dissemination complicates up to 30% cases where the metastatic growths have been found to originate from primary gastric cancer [21, 22]. While systemic chemotherapy improves median survival for patients with metastatic gastric cancer to 7-10 months [23], the same level of improvement has not been observed in patients with PC from gastric cancer [24]. As such, localized therapy is very important for these patients as a high intraperitoneal concentration of drugs will have better access to the tumors compared with systemic administration [25, 26]. Ideally for intraperitoneal chemotherapy, the drugs should escape the peritoneal cavity slowly, so the drugs have the maximum chance to penetrate into the tumor [27].

Recently, DTX was used in intraperitoneal chemotherapy. The combined chemotherapy of S-1 plus intraperitoneal DTX was found to be safe and effective for gastric cancer with peritoneal dissemination [10]. As such, in this work we used the same treatment for cancers with low-level expression of TUBB3.

According to previous research, intravenous chemotherapy has little effect on PC [8, 28]. While intraperitoneal chemotherapy was shown to be partially effective, the response rate was less than 30% [29-31]. In this work, using gene expression levels to select the chemotherapy drug, we combined intraperitoneal and oral chemotherapy, and demonstrated clinical regression of ascites and related symptoms in all patients. In one case we observed complete disappearance of the ascites. All patients were classified as either PR or CR after treatment.

Previous research has shown that gene expression levels in pleural effusion and malignant ascites can be used as markers in the selection of chemotherapy drugs for treatment. For lung cancer patients with pleural effusions, EGFR mutations in the cells and cell-free DNA served as a marker for the evaluation of gefitinib therapy [32, 33]. In another paper examining the treatment of ovarian cancer patients, xeroderma pigmentosum A protein expression levels in malignant ascites was found to correlate to response from platinum-based chemotherapy [34].

In gastric cancer tissue samples, high-level expression of ERCC1 was found to correlate with platinum resistance and poor OS [35, 36], and in other studies using paraffin-embedded gastric tumors the results showed that high level TUBB3 expression meant the cancers had a poor response to chemotherapy [37]. By real-time quantitative PCR, Lu et al. [38] analyzed gastric cancer TUBB3 mRNA expression before first-line paclitaxel plus capecitabine chemotherapy. High-level TUBB3 expression of mRNA was significantly associated with a lower response rate and OS.

In this research, rather than use paraffin-embedded tumor samples, we examined malignant ascites to examine gene expression levels and then used the results to direct drug selection for chemotherapy.

For this study we chose DTX and DDP as model drugs for delivery directly into the abdominal cavity. After treatment all patients were classified as PR and CR, with the mRNA expression demonstrating a direct correlation to the chemotherapy success. The treatments were well-tolerated, effective and safe for all the patients.

In another clinical trial involving gastric cancer with PC confirmed by laparoscopy or laparotomy, the patients also received intraperitoneal chemotherapy with DTX [39]. In that trial the 1-year OS rate was 70% which was higher than the OS rate reported in this study. One reason for this may be that because those patients were diagnosis by pathology and examination by laparoscopy or laparotomy, they were not diagnosed with many malignant ascites. In contrast, all patients in our study had late stage malignant ascites, which may explain why our OS rate was shorter. In our study no patients survived beyond 2 years, and the 1-year OS rate was 40%. The results of this study are limited by the number of patients enrolled and larger cohorts should be recruited in further research. In addition, the survival benefit should be demonstrated using randomized groups. It is feasible to conduct a randomized study because unresectable peritoneal carcinomatosis of gastric origin is a common disease.

Another limitation of this study is the toxic side-effects experienced by the patients. In total, 30 cycles of chemotherapy were delivered to the patients. The grade 3/4 toxic effect was neutropenia; leucopenia was experienced only once by one patient, and one patient developed intestinal occlusion from their last cycle of intraperitoneal chemotherapy.

This treatment protocol produced a significantly long survival rate and was generally well tolerated by the patients, with particularly mild side effects. When used in combination with sensitive methods of detecting gene expression, we think that these results have application in minimizing the use of invasive treatments and could offer some advantages over current treatment methods with regard to patient outcomes and patient stay in hospital.

In conclusion, even though our results are based on a small number of patients, they suggest that monitoring of gene expression levels may be of use in directing chemotherapy for the treatment of malignant ascites that arise from unresectable peritoneal carcinomatosis. Further clinical studies, based on larger cohorts of patients are required to assess definitively the role of gene expression in directing chemotherapy.

Disclosure of conflict of interest

None.

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