Original Article

The correlation between the plasma concentration of gemcitabine and short-term efficacy and adverse reactions in patients with advanced squamous cell carcinoma of the lung using liquid chromatography-mass spectrometry

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Abstract

Background: Worldwide, non-small cell lung cancers have the highest incidence and mortality rates of all cancers. Gemcitabine (2',2'-difluoro-2'-deoxycytidine or dFdC, $C_9H_{11}F_2N_30_4$) is widely used as the first-line chemo-reagent for lung cancer patients whose tumors have been diagnosed to be at an advanced stage and are therefore unresectable.

Objective: The objective of this systematic study was to establish the correlation between the plasma concentration of gemcitabine and short-term clinical efficacy and adverse reactions in patients with advanced squamous cell carcinoma of the lung using liquid chromatography-mass spectrometry.

Material and methods: In total, 53 patients were given the chemotherapy medications, gemcitabine and cisplatin, every 3 weeks. Plasma concentrations of gemcitabine were determined using liquid chromatography-mass spectrometry. A modified methodology of the liquid chromatography-mass spectrometry system was verified and performed to detect plasma concentrations of gemcitabine. The clinical endpoints – short-term clinical efficacy and adverse reactions – were evaluated after two cycles.

Results: The plasma concentration range of gemcitabine in 53 patients was 1.58-28.70μg/ml (mean 14.37±8.63μg/ml), with 28 patients in the >15μg/ml group (mean 21.76±3.45μg/ml), and 25 patients in the ≤15μg/ml group (mean 6.09±3.57μg/ml). The clinical benefit rate (CBR) of the >15μg/ml group was significantly higher than that of the 15μg/ml group ($p<0.05$). The incidences of leukopenia and neutropenia, thrombocytopenia and grade III-IV gastrointestinal reactions in the >15μg/ml group were significantly higher than in the ≤15μg/ml group ($p<0.05$). There was no statistical difference between the two groups in terms of the incidences of reduced hemoglobin, liver and kidney function damage, allergic reaction and rash ($p>0.05$). The analysis of the plasma concentration of gemcitabine and the percentage of reduction in neutrophil count (NEUT) ($r^2 = 0.3212$; $p<0.05$) and platelet (PLT) ($r^2 = 0.6439$; $p<0.05$) showed a significant positive correlation.

Conclusions: In patients with advanced non-small cell lung cancer, a high plasma concentration of gemcitabine can improve the short-term clinical efficacy of treatment, but increase the incidence of grade III-IV adverse reactions. [*Ethiop. J. Health Dev.* 2020; 35(1):000-000]

Key words: Non-small cell lung cancer, gemcitabine, plasma concentration, short-term efficacy, adverse reactions

Introduction

Over the past five decades, lung cancer has remained the most prevalent and lethal of all types of cancer (1). The latest data, from 2019, shows that China is significantly higher than the rest of the world in terms of the morbidity and mortality of lung cancer. The Chinese population accounts for 18.6% of the global population, while morbidity and mortality from lung cancer in China accounts for 37.0% and 39.2%, respectively, of all cases across the globe (1-4). The major therapies used for lung cancer depend on the subtype. For example, patients with non-small cell lung cancer (NSCLC) mainly rely on surgery combined with chemotherapy (5, 6). When lung cancer progresses to an advanced stage, only chemotherapy can delay its rapid development (7, 8). A very small proportion of NSCLC cases in specific mutated sites can benefit from targeted drugs; the rest of the large number of patients are treated with cytotoxic reagents only (8, 9). Gemcitabine, combined with platinum-based drugs, is the first-line chemotherapeutic regimen for NSCLC (10, 11). Research shows that the response rate of NSCLC patients treated with gemcitabine combined with cisplatin is 31-54%, and the average survival time is 8.4-15.4 months, with tolerable adverse reactions (12,13).

Gemcitabine's mechanism of action is different from other antinucleotide metabolizers, such as cisplatin and fluorouracil. Gemcitabine is a novel nucleoside derivative of cytosine, Activated by deoxycytosine kinase in the human body. The double fluorinated substitution of furanose stabilizes the electron density of furanose, leading to the accumulation of fluorinated cytidine. Fluorinated cytidine is then catched by guanine to form DNA double strands, escaping exonuclease detection, thereby starting apoptosis progress (14). Given gemcitabine's broad variety of effects in a wide range of individuals, surveillance of blood concentration and monitoring of adverse events are required. (15,16). When gemcitabine came onto the market, At the early stage of gemcitabine marketing, there were reports on plasma drug concentration, but the number of research reports was few. According to the narrow safety margin of gemcitabine, related clinical studies show that there

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are significant individual differences in terms of its clinical efficacy and adverse reactions among patients. When standard doses of gemcitabine are intravenously injected, the peak of plasma concentration reaches around 10～30μg/ml (17). This wide range of effective drug concentration easily leads to unmanageable life-threatening side-effects. However, there is no integrated and standardized baseline, nor is there an effective technique for monitoring the dynamic plasma concentration of gemcitabine. Again, this is not clear. Perhaps: Thus, the monitoring of adverse events was based entirely on the self-reports of patients and observations of medical staff. But none of these options could achieve prediction of side-effects. Whether gemcitabine concentration can be used as a predictor of adverse reactions to guide individualized medication. There are several side-effects that may arise from this medication, including the suppression of bone marrow function; the loss of white blood cells, red blood cells and platelets; loss of appetite; and headaches. One particularly serious side-effect is thrombotic thrombocytopenic purpura. The use of a bone marrow stimulant while going through the drug administration and the use of antiemetic drugs can reduce the chance of associated side-effects.

Since plasma concentration is commonly used as a reference basis in clinical practice, High Performance Liquid Chromatography (HPLC) is the prevalent method for detecting plasma gemcitabine (18). Chiral column chromatography is expensive and analytical use may be restricted by poor reproducibility. A major limitation is that it requires different stationary phases for each new class of optical isomer for better separation. Since the method for isolating plasma mixture relies on specific retention time, components with the same retention time would be automatically classified as one group. This would easily introduce false positive results if the components have similar polarities. In order to precisely separate the accurate gemcitabine active substrate, liquid chromatography-mass spectrometry (LC-MS) was conducted to further separate mixtures and identify whether the component contains other impurities with the same retention time (19). The detection of serum gemcitabine using the LC-MS method has been administered in Europe and the USA, although the HPLC technology remains the major monitoring method in China. The first observation point was the feasibility of the LC-MC for the determination of Plasma concentrations of gemcitabine in Chinese. The other observation point was the correlation between plasma concentration of gemcitabine and clinical efficacy and adverse events. Besides, due to the low selectivity of gemcitabine to tumor cells, some patients experienced adverse reactions at low concentrations of gemcitabine l. Once an adverse event happens, drug withdrawal only stops the exogenous increase of gemcitabine. Those adverse events also cause additional medical expenditure.

Aiming at better sensitivity and a lower limit of detection, we managed to test whether the LC-MS method suited Chinese patients. Grade III-IV adverse events among patients treated with gemcitabine reached about 40% in our local center; the lower effective plasma level needed to be monitored, which might lead to a decline in the level of detection. In this study, we set up and modified the LC-MS technology, replacing traditional reversed-phase iron-pair HPLC to detect plasma gemcitabine in 53 lung cancer patients. The lowest threshold of detection reached 0.052ng/ml under the LC-MS system. Additionally, we integrated the distribution of the plasma level and its positive correlation with hematology adverse events.

Materials and methods

Analysis of the plasma concentrations of gemcitabine and other chemicals: Gemcitabine hydrochloride standard substances (purity: 99.8%, LOT 100622-201202, molecular weight: 263.198g/mol) and cefaclor, internal standard (purity: 95.3%, LOT 130481-201205), were purchased from the National Institutes for Food and Drug Control. All chemicals were of standard analytical grade. Acetonitrile and formic acid were purchased from Merck KGaA (Darmstadt, Germany). With respect to the toxicity level, acetonitrile is highly discouraged for use as a solvent, With respect to its toxicity level, acetonitrile is highly discouraged for use as a solvent, but it was chosen on the basis that it has a lower viscosity than methanol, which forms highly, which forms highly viscous mixtures with water at certain concentrations. Also, acetonitrile has a higher elution strength than methanol. The water purification system used was provided by Millipore Inc. (Bedford, USA).

The plasma concentrations of gemcitabine were determined by the LC-MS system. The working principle of the system involves the heat treatment of a sample mixture which separates into individual substances. Although heated gases are carried through a column with an inert gas (e.g. helium), in this study the high temperature heat treatment would reduce the efficacy of gemcitabine active compounds. Another reason of using the LC-MS method in this study on the basis of solvent selection with proper affinity.

Three main classes of test procedures can be adopted for drug resistance during treatment, after a certain period of drug administration:
1. Fresh tumor cell culture
2. Cancer biomarker tests
3. Positron emission tomography.

LC equipment and conditions: The liquid chromatography phase of plasma gemcitabine was separated using Waters Acquity UPLC H-Class system (Waters, Milford, MA, USA). Agilent ZORBAX Eclipse Plus, C18 reversed-phase column (2.1×150mm 1.8-micron) (Waters, Milford, MA, USA) was adopted and the column temperature was kept at 40℃. The mobile phase: water (containing 0.1% formic acid): acetonitrile (containing 0.1% formic acid) = 80:20. Elution rate was applied at 0.4ml/min.

Mass spectrometry equipment and conditions: A Xevo triple quadrupole (TQD) mass spectrometry with an electrospray ionization (ESI) (SCIEX, Framingham, MA, USA) source operating in the positive mode was used as a quantitative detector. For quantification,

multiple reaction monitoring (MRM) chromatograms were acquired. Optimized MS parameters included: capillary voltage 1,000V, source temperature 550℃. A retaining cone was used as 1,000 l/hr nitrogen gas and collision gas was conducted by 50 l/hr.

Standard solution and quality control (QC): The total 11.40mg standard gemcitabine was weighed twice and dissolved in 10ml methanol to get 1mg/ml stocking solution. With cefaclor as the internal standard, the internal standard method of quantitative. 10.5mg standard cefaclor was weighed twice and dissolved into a volumetric flask with 50ml solution of 10mmol/l ammonium acetate to get 0.20mg/ml cefaclor stocking solution. The working solution was diluted by the water–acetonitrile (4:1, V/V) to 8μg/ml before use. Each standard concentration (50μl) was added to 50μl blank plasma, followed by mixing with 100μl 8μg/ml cefaclor, Q500ng/ml solutions. The low (10ng/ml), medium (50ng/ml) and high (400ng/ml) QC solutions were prepared in a similar manner. The cefaclor and QC solutions were stored at 4℃.

Sample preparation: Plasma samples were gradually thawed at room temperature, and then 50μl of each sample was mixed with 100μl of internal standard working solution and 50μl of water–acetonitrile (4:1, V/V) mixture. The plasma sample mixture was then vortexed for 5 minutes at 1,250 rpm, followed by being centrifuged at 15,000rpm for 5 minutes. The supernatant was carefully collected and filtered through a 0.22μm organic membrane. The final extraction was transferred to auto sampler vials with inserts for loading analysis. All samples were stored at −80℃ until further use.

Method validation: We followed the guidelines of the US FDA Guidance for Industry Bioanalytical Method Validation, as well as guidelines produced by the European Medicines Agency and Chinese Pharmacopoeia (2015).

The validation of the method was carried out for sensitivity, selectivity, standard cure and low of limit, precision and recovery rate, matrix effect, residue effect, continuous calibration (CC) standards and QC samples.

Specificity: 50μl each of standard gemcitabine solution series and blank plasma sample were mixed with an additional 50μL of (water:acetonitrile = 4:1), vortexed for 30 seconds, and centrifuged for 5 minutes at 1,5000 rpm. The supernatant was passed through a 0.22μm organic filter, and 5μl was taken for simultaneous injection, and the chromatogram results were recorded.

Investigation focused on whether the samples, internal standard, and plasma substances could be completely separated without interference of gemcitabine.

Standard curve and low of limit: The chromatogram results of each standard solution were recorded. The peak area ratios of gemcitabine and the internal control (Cefaclor) were plotted as ordinate (y-axis), and the concentration of gemcitabine was plotted as abscissa (x-axis). Next, the linear regression between these two factors was analyzed, thereby obtaining standard curve and low of limit.

Recovery and precision: Three QC solutions with concentrations of 10, 50, 400ng/ml^{-1} were prepared using the process indicated above. Each concentration had five repetitions, and each sample was determined continuously in three days in order to calculate the inter- and intra-day precision and recovery.

Matrix effects: Six plasma samples of the same concentration were prepared, three with matrix and three without matrix. The peak ratio of matrix with matrix-free was calculated. The ideal ratio should be within the 85%-115% range.

Residual effects: High QC solutions and five plasma samples with different batches were alternatively injected to determine whether any residue existed after injecting the high QC solution.

Stability: Low, medium and high QC working solutions were placed at room temperature and 4℃, respectively. Re-determination of concentration for each sample was conducted at 0, 4, 8, 12 and 24 hours. The change of concentration in each solution that were stored for 90 days at –20oC and 180 days at –80oC was conducted as freeze-thaw stability.

Patient enrolment: Eligibility criteria included:

1. Patients with cytological or pathological diagnosed advanced lung squamous cell carcinoma (III B/IV)
2. Medical records ranging from January 2017 to June 2018 in the people's hospital of Guangxi Zhuang autonomous region
3. 18-75 years of age
4. Eastern Cooperative Oncology Group(ECOG) score ≤2
5. Expected survival time >3 months
6. Measurable lesion ≥1 and no brain metastases
7. No gastrointestinal diseases and symptoms before chemotherapy
8. No cardiac, hematological, liver or renal function anomalies
9. No disequilibrium of basic metabolism
10. No radiotherapy or receiving radiotherapy after 6 weeks.

Exclusion criteria included:

1. Chemotherapeutic cycle <2
2. Preventive conduction of granulocyte colony stimulating factor (G-CSF) or pharmacologically similar drugs before chemotherapy
3. Concurrent combination of radiotherapy.

All of the enrolled patients signed informed consent forms, and the study was approved by the ethics committee of the people's hospital of Guangxi Zhuang autonomous region.

Therapeutic protocol: All patients received intravenous injections of 1,000mg/m^2 gemcitabine (Gemzar®, LOT676406, Eli Lilly and Company, Indianapolis, USA) and 75mg/m^2 cisplatin (LOT130701, Hansoh Pharma, Jiangsu Province, China). Freeze-dried

gemcitabine was diluted with 100ml 0.9% NaCl solution before use and then slowly dripped within 30 minutes on day 1 and day 8 of the therapy cycle, cisplatin was diluted in 500ml 0.9% NaCl solution before use and then slowly dripped within 60 minutes on day 1 of the therapy cycle.

Sample collection: For each patient, a total of 2-3ml of venous blood was collected in EDTA-anticoagulated tubes within 5 minutes of finishing the infusion of gemcitabine. All samples were labeled and immediately placed in an ice water bath and transferred to the laboratory. Plasma was then separated by centrifuge (Thermo X3R) at 4℃ at 3,000 rpm for 30 minutes. Each plasma sample was stored at –20℃ before determination. The determination was conducted within one week.

Observation target and evaluation: Baseline evaluation was integrated through manifestations and objective examination. Patients' manifestations contained the aspects of gender, age, weight, pathological type, ECOG PS, chest X-ray, CT and other imaging data. Laboratory examination included blood routine examination, liver and kidney functions, and cardio functions. After two cycles of chemotherapy, the short-term clinical efficacy of the patients was evaluated by measuring the tumor focus based on imaging data. Adverse side-effects, including gastrointestinal reaction, myelosuppression, liver and kidney injury, allergic reaction and skin rash, were recorded and evaluated at the end of each cycle. The percentage reduction in neutrophil count and platelet (PLT) was calculated as follows:

NEUT and PLT (%) = ×100%.

$$\frac{\text{count of baseline} - \text{count after chemotherapy}}{\text{count of baseline}}$$

The short-term clinical efficacy was measured by Objective Response Rate (ORR) and clinical benefit rate (CBR). It was classified as complete response (CR), partial response (PR), stable disease (SD) and progression of diseases (PD), based on the response evaluation criteria for solid tumor therapy (RECIST) (8).

$$\text{ORR} = \frac{CR + PR}{CR + PR + SD + PD} \times 100\%, \quad \text{CBR} = \frac{CR + PR + SD}{CR + PR + SD + PD} \times 100\%.$$

Adverse reactions were determined as the most severe reaction after each cycle of chemotherapy and recorded in the analysis as statistical data. The classification was carried out according to the classification standards for acute and subacute toxic and side-effects of anticancer drugs.

Statistical analysis: Data are presented as mean ± SD (median); all data were analyzed for descriptive statistics using SPSS version 22 (IBM, USA); the plasma concentrations were compared with a paired Student's t-test; the adverse reaction and short-term clinical efficacy rates were compared with a chi-square test, the level of significance of which was set at $p<0.05$.

Results: Method validation

Specificity: The LC-MS system was used to test the retention time of gemcitabine and the internal control, cefaclor (cefaclor is a second generation cephalosporin antibiotic, extensively used to treat bacterial infections of respiratory tract, skin, ears, throat, tonsils and urinary tract. Molecular formula: $C_{15}H_{14}ClN_3O_4S$; molecular weight: 367.8g/mol). cefaclor was plotted as ordinate (y-axis), and the concentration of gemcitabine was plotted as abscissa (x-axis). Linear regression analysis was carried out for further interpretation. Endogenous substrate of plasma did not interfere with either gemcitabine or cefaclor, thereby proving the good specificity of the LC-MS method.

Standard curve and low of limit: The standard curve for gemcitabine in plasma was linear over the range 5ng/ml to 500ng/ml; the standard curve's regression was C=1272.93A+11213.5, r = 0.999. The lower limit of quantitation for gemcitabine and QC cefaclor in plasma was 0.052ng/ml.

Recovery and precision: The RSD (recovery and relative standard deviation) value of recovery of precision among low, medium and high QC solutions ranged from 89.41% to 101.42%. The intra-day RSD and inter-day RSD of these three QC solutions were 4.55%, 3.09% and 2.81% vs 6.92%, 5.22% and 3.63% (see Table 1).

Table 1: **Precision and recovery of gemcitabine in plasma**

Concentration	Intra-day RSD (%)	Inter-day RSD (%)	absolute recovery (%)
10	4.55	6.92	89.41
50	3.09	5.22	98.38
400	2.81	3.63	101.42

Matrix and residue effects: The matrix effects results of low, medium and high QC solutions fluctuated around 92-108%, and the high QC solution had no residue according to its chromatogram. These results indicated that the matrix could not disturb the results and the method would not cause residue effect.

Stability: Three QC solutions obtained good short-term routine storage and long-term cryopreservation. The RSD value of each determination of concentration was under 20%, as illustrated in Table 2.

Table 2: Stability of gemcitabine in plasma

Concentration	Room temperature RSD (%)	2-4°C RSD (%)	–20°C RSD (%)	–80°C RSD (%)
10	4.26	4.78	11.67	14.33
50	4.17	3.96	10.46	16.52
400	2.36	2.85	7.98	13.87

Results: Demographic information and clinical baseline

Fifty-three patients were enrolled in this study: 33 males and 20 females. Their ages ranged from 23 to 75 years, averaging 54.1±10.96 years old. For all the participants, the median plasma concentration of gemcitabine was 15μg/ml, which further was treated as a cut-off value to classify high (>15μg/ml) and low (≤15μg/ml) concentration groups. Details of the demographic information of participants are in Table 3. There was no statistical difference between the two groups in terms of age, gender, body mass index (BMI), pathological type, disease stage and ECOG score ($p>0.05$).

Table 3: Comparison of demographic information of the two groups

	>15μg/ml group	≤15μg/ml group	X^2	P
Total case	28	25		
Average age	55.7±9.0 years	53.22±11.2 years	0.428	0.527
Gender (male:female)	17:11	16:9	0.391	0.412
BMI (kg/m^2)	21.83±5.83	23.16±4.26	0.097	0.785
Pathological type (adenocarcinoma /squamous cell carcinoma/others)	19/8/1 (67.86%/28.57%/3.57%)	16/8/1 (64.00%/32.00%/4.00%)	0.476	0.469
Tumor stage (III B/IV)	10/18 (35.71%/64.29%)	8/17 (32.00%/68.00%)	1.247	0.264
ECOG PS (0/1/2)	18/8/2 (64.29%/28.57%/7.14%)	17/8/0 (68.00%/32.00%/0.00%)	1.683	0.192

Results: Distribution of plasma gemcitabine

The distribution of plasma gemcitabine concentration among 53 patients complied with non-normal distribution. The plasma gemcitabine concentration ranged from 1.58μg/ml to 28.70μg/ml, with an average concentration of 14.37±8.63μg/ml, as shown in Table 4 and Figure 1.

Table 4: **Determination of gemcitabine in human plasma**

Sample n	concentrations (μg/ml)	Sample n	Concentrations (μg/ml)	Sample n	concentrations (μg/ml)	Sample n	concentrations (μg/ml)
1	1.58	15	3.82	29	18.78	43	22.07
2	1.96	16	7.93	30	24.27	44	19.16
3	2.06	17	7.52	31	16.85	45	28.7
4	2.18	18	7.91	32	20.76	46	25.48
5	2.54	19	8.04	33	24.93	47	19.39
6	3.17	20	8.21	34	23.84	48	27.18
7	3.68	21	9.76	35	22.17	49	19.05
8	4.08	22	11.06	36	25.78	50	23.84
9	4.96	23	13.78	37	21.46	51	17.32
10	5.78	24	14.89	38	27.36	52	16.81
11	6.13	25	5.81	39	19.26	53	21.83
12	6.82	26	15.76	40	24.14		
13	3.93	27	19.84	41	20.36		
14	4.71	28	19.84	42	23.14		

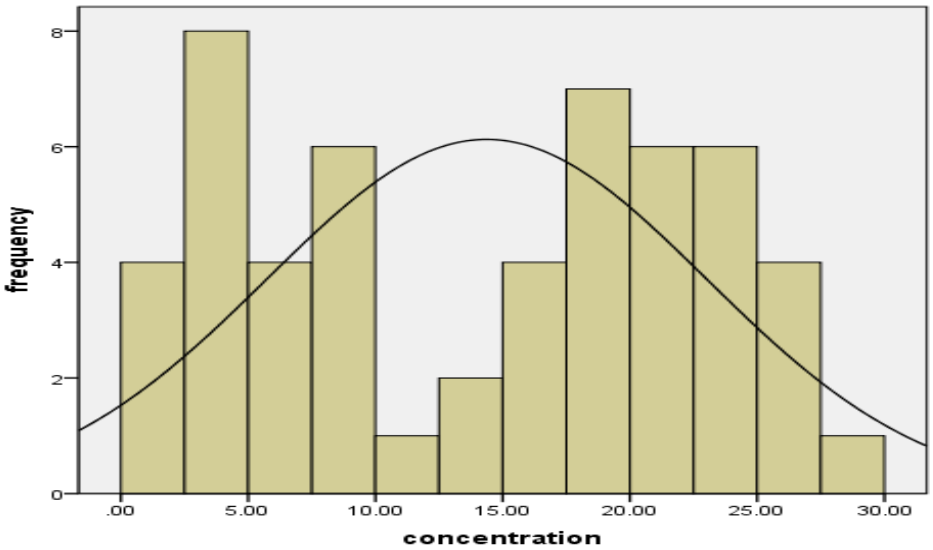

Figure 1: **Distribution of plasma concentration of gemcitabine among participants**

Results: Relationship between plasma gemcitabine and short-term clinical efficacy

The clinical efficacy was evaluated as per the RECIST guideline. The disease response rates are listed in Table 5. The high plasma gemcitabine concentration group (>15μg/ml) showed a better CBR score of 85.71% compared with 76.00% for the low plasma gemcitabine concentration group (≤15μg/ml) (p = 0.036), which implies that patients with a plasma gemcitabine concentration over 15μg/ml might obtain promising clinical benefits. There was a similar tendency between two groups in relation to the ORR score – the high plasma gemcitabine concentration group (>15μg/ml) scored a higher ORR (35.71%), whereas the lower score of 28.00% was illustrated in their counterparts, with no statistical significance.

Table 5: **The relationship between the concentration of gemcitabine and short-term efficacy**

Concentration	CR	PR	SD	PD	ORR	CBR
>15μg/ml	3 (10.71%)	7 (25.00%)	14 (50.00%)	4 (14.29%)	35.71%	85.71%
≤15μg/ml	2 (8.00%)	5 (20.00%)	12 (48.00%)	6 (24.00%)	28.00%	76.00%
x^2	-	-	-	-	2.533	4.248
P	-	-	-	-	0.053	0.036

Note: ORR: Objective Response Rate; CBR: clinical benefit rate; CR: complete response; PR: partial response; SD: stable disease; PD: progression of diseases.

Results: High plasma gemcitabine predicted particular adverse events

Although high plasma gemcitabine relates to better clinical efficacy, it was unknown if it was related to any consequent adverse events. Therefore, all of the enrolled subjects were under medical surveillance any adverse events occurred during the first two cycles. Gastrointestinal reactions remained the most prevalent adverse events in both groups, with an incidence of 82.14% in the high plasma gemcitabine group and 72% in the low plasma gemcitabine group, regardless of the severity grade. Patients with low plasma gemcitabine had a slightly higher proportion of the occurrence of mild side-effects (level I-II) in terms of gastrointestinal reactions, liver injury and renal function, though none of them showed statistical significance (p>0.05, see Table 6).

Table 6. The relationship between concentration of gemcitabine and adverse reactions

Adverse reactions		n	Level I-II		x^2	P	Level III-IV		x^2	P
			n	%			N	%		
Gastrointestinal reaction	>15μg/ml	28	17	60.71	0.370	0.642	6	21.43	4.26	0.032
	≤15μg/ml	25	16	64.00			2	8.00		
Hepatic injury	>15μg/ml	28	4	14.29	0.028	0.868	1	3.57	0.36	0.739
	≤15μg/ml	25	4	16.00			1	4.00		
Renal function impairment	>15μg/ml	28	2	7.14	1.562	0.307	0	0	-	-
	≤15μg/ml	25	3	12.00			0	0		

For common chemotherapeutic conditions such as skin rash, influenza-like symptoms, anaphylaxis and alopecia, there was no obvious difference between the two groups (see Table 6). In terms of higher grade side-effects (level III-IV), these were found in around one fifth of patients in the high plasma gemcitabine group and in 8% of patients in the low plasma gemcitabine group (see Table 7). The statistical difference between the two groups in terms of the incidence of level III-IV gastrointestinal reactions was significant ($p<0.05$)

Table 7. The relationship between concentration of gemcitabine and other major adverse reactions

Adverse reactions	N	Skin rash		Influenza-like symptoms		Anaphylaxis		Alopecia		Others	
		n	%	n	%	n	%	n	%	n	%
>15μg/ml	28	3	10.71 %	2		2	7.14 %	4	14.29 %	1	3.57%
≤15μg/ml	25	2	8.00%	2		2	8.00 %	4	16.00 %	1	4.00%
x^2	-	0.18		0.274		0.764		0.417		0.532	
P	-	0.667		0.752		0.367		0.623		0.455	

In addition, we found that all participants suffered different levels of chemotherapy-related myelosuppression, which leads to irreversible and lethal events. The high plasma gemcitabine group had a higher incidence of induced leucopenia, granulocytopenia and thrombocytopenia compared to the low plasma gemcitabine group at all grades of severity. Chi-square analysis demonstrated that the difference of incidence between the two groups was statistically significant ($p<0.05$).

Since the obvious higher possibility of chemotherapy-related myelosuppression occurred among patients with high plasma gemcitabine, we assumed that the severity of chemotherapy-related myelosuppression was induced by an increase in plasma gemcitabine. According to the evaluation assessment, the severity of myelosuppression was positively related to the reduction in their plasma level. Spearmen correlation analysis was conducted to determine the correlation between plasma gemcitabine and the reduction of plasma level of NEUT and PLT, respectively. The increased reduction of NEUT and PLT was positively correlated with higher plasma gemcitabine. Figure 2A and 2B show the correlation between NEUT, PLT and the plasma concentration of gemcitabine individually. The specific coefficient r^2 for NEUT reduction was 0.827 ($p<0.05$), and 0.578 ($p<0.05$) for PLT reduction (see Table 8), which instructed the higher plasma gemcitabine reflected the higher the incidence of myelosuppression. Based on these data, the surveillance of plasma gemcitabine could be used as a predictor of adverse reactions to guide individualized medication.

Table 8: **The relationship between concentration of gemcitabine and myelosuppression**

Adverse Reactions		**N**	**Level I**		**Level II**		**Level I-II**		χ^2	**P**	**Level III**		**Level IV**		**Level III-IV**		χ^2	**P**
			n	**%**	**n**	**%**	**n**	**%**			**n**	**%**	**n**	**%**	**n**	**%**		
Leukopenia	>15 μg/ml	28	7	25.070	5	17.86	12	42.86	5.13	0.029	4	14.29	3	10.71	7	25.00	4.42	0.031
	≤15 μg/ml	25	5	20.050	3	12.00	8	32.00			2	8.00	2	8.00	4	16.00		
Granulocytopenia	>15 μg/ml	28	7	25.070	3	10.71	10	35.71	4.33	0.046	3	10.71	3	10.71	6	21.43	4.87	0.049
	≤15 μg/ml	25	5	20.050	2	8.00	7	28.00			2	8.00	2	8.00	4	16.00		
Decreased Haemoglobin	>15 μg/ml	28	4	14.249	2	7.12	6	21.43	0.25	0.628	3	10.71	1	3.57	4	14.29	0.27	0.794
	≤15 μg/ml	25	3	12.030	2	8.00	5	20.00			2	8.00	1	4.00	3	12.00		
Thrombocytopenia	>15 μg/ml	28	6	21.463	3	10.71	9	32.14	9.11	0.004	3	10.71	3	10.71	6	21.43	4.22	0.022
	≤15 μg/ml	25	3	12.030	2	8.00	5	20.00			2	8.00	1	4.00	3	12.00		

Different anticancer drugs are used to treat different types of tumors, with different drug categories affecting abnormal cells in many ways. They have different origins and target component cells and have side-effects on the human body. The treatment regime of anticancer drugs is such that drugs are given for a set time duration at repeated intervals. Chemotherapy drugs may be given according to different schedules. In Table 9 we summarize other anticancer drugs and corresponding cell lines used.

Table 9: **List of generic oncology products, originator products, and the corresponding tumor cell lines used**

Generic oncology products	Originator	Tumor cell line used	Origin
Paclitaxel	Taxol	MCF-7	Breast carcinoma
		NCI-H2126	Non-small cell lung carcinoma
Docetaxel	Taxotere	MCF-7	Breast carcinoma
		SKOV-3	Ovarian carcinoma
		PC-3	Prostate carcinoma
		NCI-H2126	Non-small cell lung carcinoma
Oxaliplatin	Eloxatin	HT-29	Colorectal carcinoma
Bicalutamide	Casodex	PC-3	Prostate carcinoma
Anastrozole	Arimidex	MCF-7	Breast carcinoma

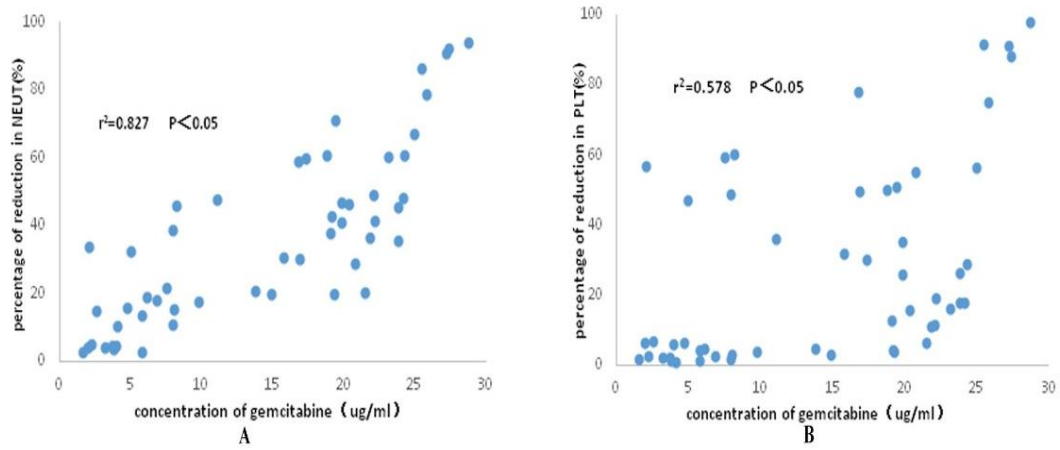

Figure 2: **The correlation between NEUT, PLT and plasma concentration of gemcitabine**

Discussion

We chose gemcitabine over other drugs, such as cisplatin and fluorouracil, because related clinical studies indicate its wide range of acceptability among patients, given its easy penetration into cell DNA compared to other drugs. The optimum concentration for application is still under study. First, we considered two problematic issues: the condition of traditional RP-liquid chromatographic methodology with an extremely short retention time for weak basic compounds; and the complicated mixture of human plasma, which easily interferes with the accuracy of separation (20). Due to the basic deoxycytidine structure of gemcitabine, its extremely strong polarity makes it difficult to retain in a normal chromatographic column (20,21). Therefore, in this study, the column that applied in liquid chromatography was replaced by ZORBAX Eclipse PlusC18, which was a modified porous RP-C18 silica gel with ultra-high purity level through chemical bonding dimethyl-n-octadecylsilane to ZORBAX Rx-SIL silica gels (Class B). Silica column normal was conducted for separating acidic and neutral sample, since the basic compounds would form tight bonds with padding. However, the ZORBAX modified silica column can reduce and eliminate the strong adhesion of basic and highly polar compounds through covalent binding with octadecylsilane (22). Therefore, this modified chromatographic column was widely applied to separate alkaline with increasing accuracy.

As for reducing the interference of the plasma mixture, the protein precipitation was a key step for filtrating the major impurity, thereby improving the chromatographic accuracy (23). Plasma contains various proteins with polarity affecting pH value; their amino acid residue normally carries different charges which could disturb the chemical's retention. Besides, the large molecular weight of protein blocks the filtration of the impurity. In the preliminary experiment, three combinations of organic reagents were used for precipitating plasma protein: 30% trichloroacetic acid and water solution, methanol and acetonitrile (1:9 V: V) solution, and water and acetonitrile (4:1 V: V) solution. The results showed the sample peak and its tailing factor were dramatically improved, as well as increasing the signal response of gemcitabine under pre-treatment with the water and acetonitrile (4:1 V:V) solution compared to the other two precipitated solutions. Although these three combinations were all certified protein precipitation reagents, in our system, the water and acetonitrile mixture achieved the most optimal exclusion of plasma protein.

We also modified the condition of the mobile phase with a slightly decreasing pH value to obtain a better signal response value of both gemcitabine and cefaclor. Four mixtures containing different weak acid concentrations were applied for candidate elution: A. 0.52% sodium dihydrogen phosphate solution (pH 2.66) and acetonitrile (85:15, containing 0.202% sodium heptane sulfonate); B. 0.01% acetic acid water and acetonitrile (80:20); C. acetonitrile: 0.1% trifluoroacetic acid (3:97); and D. 40 mol/l ammonium acetate buffer and acetonitrile (97.5:2.5). Elutions A and B decreased the

tailing effects and increased the signal response value of both gemcitabine and cefaclor, which reflected in a better and specific peak shape in their chromatogram. Given the inherent properties of gemcitabine, an additional weak acid component could eliminate the uneven tight bond between it and column padding, resulting in a constant stable eluant velocity. However, the pH value also restricts at small scale, since the dramatic change in ion strength, compound dissociation and charge equilibrium would neutralize gemcitabine and interrupt the eluent order.

Therefore, the administration of methodology in our LC-MS system was optimal for testing the plasma concentration of gemcitabine (24). The plasma concentration of gemcitabine in 53 patients had a positive correlation with short clinical efficacy as well as severe myelosuppression. Except for myelosuppression, high and low plasma concentrations of gemcitabine had no obvious difference in organ function, such as liver, kidney and heart, nor in common indices, such as gastrointestinal reaction, skin-related symptoms and anaphylaxis. Our results showed a high incidence of gemcitabine-related myelosuppression among NSCLC patients; Tian *et al.* reported similar results of major side-effects (25). A similar research study of 82 patients also indicated that nearly 25% of pancreatic cancer patients suffered level III-IV myelosuppression after administering gemcitabine (26). A higher incidence of gemcitabine-related myelosuppression was also observed among solid tumor patients with nicotine accumulation (27).

To avoid non-tumor-related deaths, dose reduction or drug withdrawal was normally applied when severe side-effects occurred. This intervention led to a drop in the plasma concentration of gemcitabine, thereby attenuating clinical short-term efficacy. Though the range of effective plasma concentrations of gemcitabine is unclear, a low concentration of gemcitabine might accelerate the metabolism of tumor cells, which furthered activate the proliferative signal of tumors (7,28). If patients could tolerate side-effects, increasing the dose to the peak level relative to the high level of plasma concentration might improve their short-term clinical efficacy. The good tolerance in the first two cycles might aggrandize patients' compliance. Also, a constant high level of gemcitabine might cause resistance towards normal cells, due to the hyposensitization of continuous strong activation from gemcitabine (29).

The side-effects of chemo-reagents should be prevented when they can be predicted. In order to decrease gastrointestinal reaction, therapy involved the high possibility of causing nausea or vomiting was suggested with addition of antiemetic drugs at the initiation of chemo-cycle especially combine with cisplatin reagent (30). According to our results, we assumed the surveillance of plasma concentration of gemcitabine and the premonitory symptom of myelosuppression called up exogenous supplement of colony-stimulating factor, since the timing administration of bone marrow stimulant might compensate for the slight myelosuppression (31,32).

Conclusions

The modified LC-MS methodology was suitable for detecting the plasma concentration of gemcitabine. The plasma concentration of gemcitabine was positively associated with adverse reactions and short-term curative effects in patients with advanced NSCLC. High plasma concentrations can improve the short-term clinical efficacy of gemcitabine treatment, but increase the incidence of grade III-IV adverse myelosuppression-related events.

The research team for this study is planning to prolong the clinical observation and incorporate the survival rate, so that long-term efficacy can be evaluated. As metabolized gemcitabine suppresses tumor growth, if the metabolized type could be tested in parallel with prototype gemcitabine, the relationship between metabolism and side-effects would be worthy of further investigation.

Limitations of the study

The study was limited in terms of the scale of observation, namely the small number of patients observed in a single regional medical center. Extended recruitment with specific age tiers and tumor subtypes would have resulted in more evidence to establish the population pharmacodynamics of gemcitabine.

Ethical statement

This study was approved by the people's hospital of Guangxi Zhuang Autonomous Region. Consent for data collection and publication was obtained from the patients involved in the study.

Funding sources

This study was supported by the Food and Drug Safety Research Project of Guangxi Zhuang Autonomous Region Food and Drug Administration 2016 (No: 0018), and the Guangxi Zhuang Autonomous Region Health and Family Planning Commission, Guangxi Medical and Health Self-financing Program (No.: z2016610).

Authors' contributions

Jia-xi Xi: Study design and conduction; Hua-jun Zhang: Writing and editing; Xiao-yu Chen : Data collection; Dong-mei Ye: Sample process; Bi-quan Lan: Sample collection; Ying Chen: Data management and analysis; Heng-hai Su: LC-MS sampling and operation.

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