RESEARCH ARTICLE

DNA Repair Capacity in Peripheral Blood Lymphocytes Predicts Efficacy of Platinum-based Chemotherapy in Patients with Gastric Cancer

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Abstract

Objective: To investigate the correlation between ERCC1 expression levels in tumor tissue and peripheral blood lymphocytes (PBL) from patients with gastric cancer and assess the relationship between PBL DNA repair rate (DRR) and the efficacy of platinum chemotherapy. Methods: A total of 53 patients with gastric cancer receiving surgery and 20 controls were studied. ERCC1 protein expression in tumour tissue and PBL were determined by immunohistochemical staining. The PBL DRRs of 47 advanced patients and 20 controls were estimated by comet assay. Results: The positive expression rates of ERCC1 were 67. 9%, 56. 6% and 10.0% in tumour tissues, PBLs of gastric cancer patients, and PBLs of the control group. PBL ERCC1 expression correlated with that in tissue (χ^2=15. 463, *p*=0.000). Pearson contingency coefficient=0.475). DRRs of cancer patients by tail length (TL) (Z=4. 662, *p*=0.000) and tail moment (TM) (Z=3. 827, *p*=0.000) were significantly lower than that of control group. When TL was applied as an indicator, the correlation between DRR and chemotherapy efficacy was significant (Spearman rank correlation r=0.327, *p*=0.032). Patients with low levels of DRR in PBL presented better short-term efficacy of chemotherapy than those with high levels of DRR. Conclusions: The ERCC1 expression in PBLs may indirectly reflect ERCC1 expression in gastric cancer tissues. Compared with non-cancer populations, patients with gastric cancer may have lower DNA repair capacity. DRR in PBL may predict the short-term efficacy of platinum-based chemotherapy for patients with advanced gastric cancer.

Keywords: Stomach neoplasms - DNA repair - lymphocytes - platinum - comet assay - immunohistochemistry

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Introduction

Gastric cancer remains the second leading cause of cancer death, with an estimated three hundred thousand deaths in China every year. Most of gastric cancer patients are treated with chemotherapy, particularly for advanced cases, but the treatment efficacy is still poor. Recently, many studies have been done on Individualized therapy by selecting patients who are likely to respond to a particular chemotherapeutic regimen, and this may allow improved treatment efficacy while avoiding unnecessary treatment side effects.

Platinum is an significant drug in gastric cancer chemotherapy. It causes platinum-DNA adducts that block transcription, leading to cytotoxicity and cell death. Nucleotide excision repair (NER) is one of several DNA repair pathways for correcting the DNA structures that arise from DNA damage (Rabik and Dolan, 2007). Excision repair cross-complementation 1 (ERCC1) is one of the key enzymes in the NER pathway (Niedernhofer et al., 2004). As shown by most studies, both ERCC1 mRNA and protein expression are negatively correlated with the efficacy of platinum (Matsubara et al., 2008; Ozkan et al., 2010).

At present, analyses of ERCC1 expression are mainly based on tumor tissue samples from operative resection or gastroscopy. And these bring pain to the patients. Thus, there is a need to develop affordable and non-invasive methods that can be used to detect ERCC1 expression conveniently. Many studies have confirmed that peripheral blood lymphocytes (PBL) and tumor cells carry the homologous gene (Kaspers et al., 1991; Yang et al., 2006). If a correlation between ERCC1 expression in tissue and blood was discovered, it would greatly advance the development of clinical practice.

Furthermore, other DNA repair enzymes and mechanisms relate with platinum-based chemotherapy sensitivity, such as ERCC2, X-ray repair pathway (McGurk et al., 2006; Wang et al., 2012). However, above repair genes only reflect DNA repair capacity in one stage of the complex mechanisms. A phenotypic DNA repair marker that may represent the sum of all genetic variants is desirable. Currently, the DNA repair rate (DRR), as an indicator, can represent the individual DNA repair capacity

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in comet assay (Zheng et al., 2005). If DRR could predict platinum efficacy as a sensitive marker, it would be more accurate for drug choice.

Therefore, the aims of the present study were to evaluate the correlation between the ERCC1 expression of gastric cancer patients in tumor tissue and PBL, and to evaluate the correlation between the DRR in PBL of advanced gastric cancer patients and the efficacy of platinum-based chemotherapy.

Materials and Methods

Patients and Treatment

Patients treated in the medicine oncology department of the First Affiliated Hospital of Anhui Medical University from Feb 2012 to Dec 2012 were screened at gastric cancer diagnosis.

Inclusion criteria of patients who evaluated the correlation between the ERCC1 expression in tumor tissue and PBL were: (1) histologically confirmed gastric cancer by surgery; (2) no previous chemotherapy or radiotherapy; (3) integrity of clinical and pathological data; (4) signed informed consent documents prior to entering the study. And inclusion criteria of patients who evaluated the correlation between DRR in PBL and the efficacy of platinum-based chemotherapy were: (1) histologically confirmed gastric cancer; (2) no previous chemotherapy or radiotherapy; (3) integrity of clinical and pathological data; (4) signed informed consent documents prior to entering the study; (5) Eastern Cooperative Oncology Group performance status (ECOG-PS) < 3; (6) had measurable lesion; (6) received two cycles of chemotherapy with cisplatin (20 mg/m^2 D1-5) /oxaliplatin (100 mg/m^2 D1) and fluorouracil (capecitabine 1. 0 Bid D1-14/tS-1 40 mg Bid D1-14) at least.

The response of advanced gastric cancer patients was evaluated after every two cycles of therapy according to the Response Evaluation Criteria in Solid Tumors (RECIST).

In addition, peripheral blood of 20 cases of non-cancer patients were collected as a control group.

Separation of PBL

Blood samples were collected in heparin sodium-containing tubes from patients before chemotherapy and control group. PBLs were isolated from the whole blood on histopaque gradients (Histopaque 1077, TBD, Tianjin, China).

Immunohistochemistry

Paraffin-embedded tumor material from the biopsy was cut into 4 μm-thick sections and placed onto glass slides. And then the slides were depafaffinized in xylene. for epitope retrieval, specimens were exposed to 10 mM citric acid antigen retrieval solution (pH 6, 0) and heated for 30 minutes in a water bath.

PBLs were placed onto glass slides and fixed with methanol. Triton X-100 penetrated membranes of PBL.

Tumor (PBL) sections were incubated for 1 night with a monoclonal antibody specific against the human ERCC1 protein (mouse, clone 8F1, Maixin Bio, Fujian, China). Antibody binding was detected by means of an Maxvision kit (sheep/rabbit anti-mouse, Maixin Bio, Fujian, China) for 15minutes as the substrate and hematoxylin as the counterstain. Sections of esophageal squamous carcinoma tissues were included as positive controls. PBS was instead of ERCC1 antibody as negative controls. Two pathologist who were unaware of clinical data independently evaluated the percentage of positive tumor nuclei under a light microscope at a magnification of 400x. The grading system was as follows: If immunoreactivity was noted in less 10% of the tumor cells (PBLs), then we defined this as negative; If the immunoreactivity was 10% or more of the tumor cells (PBLs), then we defined this as positive (Wachters et al., 2005).

Comet Assay

The PBLs were suspended in PRMI 1640 medium (GIBCO, USA, excluding fetal calfserum), then treated with cisplatin (20 μg/mL) for 30 minutes. Then the cells were washed two times with PBS, suspended in PRMI 1640 medium (GIBCO, excluding fetal calfserum) in the dark for 15 minutes for DNA repair. Cells were tested for viability using the trypan blue dry exclusion technique. Only cell samples whose viability was over 90%, were measured by comet assay. All reagents of comet assay were from Sigma-aldrich (USA).

The solution containing 0.5% normal melting agarose (NMA) and 0.5% low melting agarose (LMA) was prepared in Ca^{2+}, Mg^{2+} free PBS. Cells with and without cisplatin treatment were suspended in LMA, and 85 μL was pipetted onto a frosted glass microscope slide pre-coated with an 110 μL layer of 0.5% NMA. The third layer of 85 μL of 0.5% LMA was added finally. Then the slides were immersed in ice-cold freshly prepared lysis solution (1% N-lauroylsarcosine sodium salt, 2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris-HCl, 1% Triton X-100 and 10% DMSO, pH=10) to lyse the cell proteins and allow DNA unfolding. After at least 1 h at 4°C in the dark, the slides were covered with fresh buffer (1 mmol/L Na_2EDTA, 300 nmol/L NaOH, pH >13) in a horizontal electrophoresis unit. The slides were allowed to sit in this buffer for 20 minutes for DNA unwinding. Then, the DNA was electrophoresed at 25V and 300mA for 20min. Both unwinding and electrophoresis were performed at an ambient temperature of 4°C. The slides were washed gently to remove alkali, deterged in a neutralization buffer (0.4 mol/L Tris-HCl, pH=7. 5) and placed in methanol for 3 min, then stained with 50μL ethidium bromide (20 μg/mL). All steps described above were conducted under yellow light or in the dark, to prevent additional DNA damage. The pictures of 50 cells per sample were taken individually under a fluorescence microscope (Olympus, Japan) and digital camera (Canon, Japan) at 400 × magnification. Tail length and tail moment were analyzed using Comet Assay Software Project (CaspLab, UK).

Individual DNA repair capacity was evaluated by DNA damage situation of pre-cisplatin treatment and following 15 minutes' repair . TL and TM ceiling of 95% of control group sample cells were seen as the standard boundaries of damaged cells and not damage cells. DRR (%) = (cisplatin treatment undamaged cells/total detected cells)/

Table 1. The Relationship Between Characteristics of 53 Patients with Gastric Cancer and ERCC1 Expression

Characteristics	Number	Percentage (%)	ERCC1 positive cases in PBLs	$\chi^2(p)$	ERCC1 positve cases in tumor tissues	$\chi^2(p)$
Age(years)	24-80					
Median(range)	59					
≥65	18	34.0	9	0.484	13	0.231
<65	35	66.0	21	(p=0.487)	23	(p=0.631)
Gender						
Male	35	66.0	19	0.225	21	2.970
Female	18	34.0	11	(p=0.635)	15	(p=0.085)
Histology						
Adeno	49	92.5	28	0.061	33	0.058
Small cell	4	7.5	2	(p>0.750)	3	(p>0.750)
Stage						
I+II	20	37.7	12	0.151	14	0.064
III+IV	33	62.3	18	(p=0.698)	22	(p=0.801)
T stage						
T1+T2	10	18.9	7	0.354	8	0.283
T3+T4	43	81.1	23	(p=0.552)	28	(p=0.595)
Metastasis lymph node	0-24					
Average	4.8					
Yes	37	69.8	21	0.001	24	0.527
No	16	30.2	9	(p=0.973)	12	(p=0.468)
Differentiation						
Poor	26	49.1	15	0.025	17	0.151
Moderate and well	27	50.9	15	(p=0.875)	19	(p=0.697)

Table 2. Baseline Characteristics of 47 Patients with Gastric Cancer

Characteristics	Number	Percentage (%)	DRR(TL) Z	DRR(TL) p	DRR(TM) Z	DRR(TM) p
Age(years)	29-80		1.508	0.132	1.672	0.095
Median(range)	62					
≥65	19	40.4				
<65	28	59.6				
Gender			0.081	0.935	0.594	0.552
Male	33	70.2				
Female	14	29.8				
Differentiation			0.488	0.625	0.228	0.820
Poor	28	59.6				
Moderate and well	19	40.4				
ECOG			0.194	0.846	1.074	0.283
1	32	68.1				
2	15	31.9				
Alcohol habits			0.674	0.500	0.832	0.405
Yes	16	34.0				
No	31	66.0				

(pre-cisplatin treatment undamaged cells/total detection cells)* 100% (Schmezer et al., 2001).

Statistical Analysis

Data analysis was performed using SPSS 18. 0 for Windows. And statistical significance was defined as p<0.05. The association between ERCC1 positive rate of gastric cancer patients and that of control group was assessed by chi-square tests. The association between the ERCC1 status and the clinical and pathological characteristics was tested for by chi-square tests. Correlation strength of ERCC1 expression in tumor tissue and PBL was assessed by the Pearson correlation test. The association between the DRR of advanced gastric cancer patients and that of control group was tested for by Mann-Whitney tests. The association between the DRR of advanced gastric cancer patients and the clinical and pathological characteristics was tested for by Mann-Whitney tests. Correlation strength of DRR and chemotherapy efficacy was assessed by the Spearman rank correlation coefficient.

Table 3. The Correlation Between ERCC1 in PBL and Tumor Tissue

Gastric cancer tissue	PBL Positive	PBL Negative	Total
Positive	27	9	36
Negative	3	14	17
Total	30	23	53

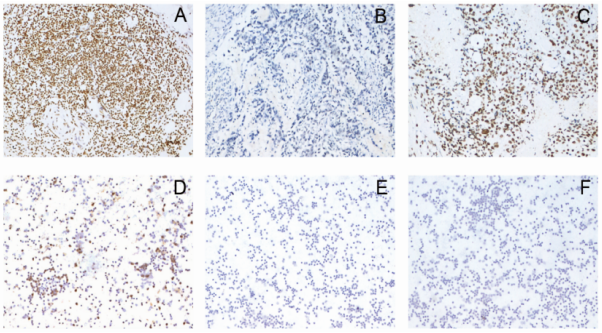

Figure 1. Typical Examples of Immunohistochemical Staining of Gastric Cancer in Representative Cases. ERCC1 shows positive immunoreactivity in tumor tissue (A) and PBL (D). In some cases, ERCC1 show negative immunoreactivity in tumor tissue (B) and PBL (E). Besides, squamous cell carcinoma tissue of the esophagus is used as positive control (C). PBLs of non-cancer people are served as negative controls (F). (all figures, *100)

Results

Patient characteristics

A total of 53 gastric cancer patients were recruited to compare ERCC1 expression in tissue and PBL. The baseline characteristics of the 53 patients are shown in Table 1.

A total of 47 advanced gastric cancer patients were recruited to detect the association between the PBL DRR and chemotherapy efficacy. Four patients withdrew from the study due to side effects, and 43 patients' data were censored at the end of our follow-up period. The baseline characteristics of the 47 patients are shown in Table 2.

ERCC1 expression levels in tumor tissues and PBLs

The ERCC1 expression positive rates were 67. 9% (36/53), 56. 6% (30/53) and 10.0% (2/20) in tumor tissues, PBLs of gastric cancer patients, and PBLs of control group, respectively. There was no relationship between the ERCC1 expression and the factors including age, gender, histological type, clinical stage, T stage, metastasis of lymph node and degree of differentiation (Table 1). The PBL ERCC1 expression of gastric cancer patients was statistically higher compared to PBL of control group (χ^2=12.810, p<0.05).

Correlation analysis of ERCC1 in PBL and tumor tissue

A positive correlation between gene expression in PBL and gastric cancer tissue was found, and was statistically significant for ERCC1. (χ^2=15.463, p=0.000). Pearson contingency coefficient=0.475) (Table 3).

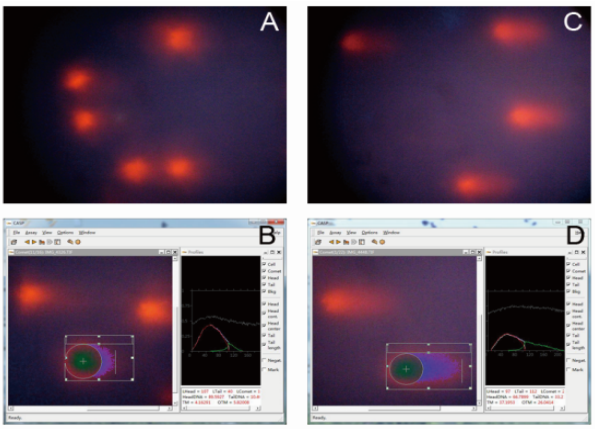

Figure 2. PBL Comet of pre-DDP Treatment (A, B) and Post-DNA Repair (C, D). CASP is Used to Analyse Comets (B, D). (all figures,*400)

PBL DRR of advanced gastric cancer patients measured with cisplatin treatment in vitro

When TL was served as an indicator, the range of DRRs in cancer patients was 48.39%-100.00%. Among them, median was 83.72%, quartile range was 18. 01%. And when TM was served as an indicator, the range of DRRs in cancer patients was 44.00%-100.00%. Among them, median was 87.76%, quartile range was 17.88%. In control group, when TL was served as an indicator, the range of DRR values was 89.80%-100.00%, median was 97.98%, quartile range was 4.13%. And when TM was served as an indicator, the range of DRRs in cancer patients was 88.00%-100.00%. And median was 99.00%, quartile range was 5. 52%. Moreover, the DRRs of gastric cancer patients was significantly below the DRRs of controls when both TL (Z=4.662, p=0.000) and TM (Z=3.827, p=0.000) were served as indicators.

In addition, there was no relationship between the DRR and factors including age, gender, ECOG, pathological differentiation and drinking habit (Table 2).

Correlation between PBL DRR and efficacy of platinum-based chemotherapy

43 patients completed the chemotherapy. Among them, 1 patient achieved complete remission (CR). 12 patients achieved partial remission (PR). 13 patients achieved stable disease (SD). And 17 patients achieved progressive disease (PD). Therefore, disease control rate (DCR) was 60.47%. There was no significant correlation between clinical and pathological factors such as age (χ^2=0.498, p=0.480), gender (χ^2=0.009, p=0.925), ECOG (χ^2=0.599, p=0.439), differentiation (χ^2=0.212, p=0.646), Alcohol habits (χ^2=0.371, p=0.543), and DCR. When TL was served as an indicator, DRR medians of CR, PR, SD and PD patients were 74. 00%, 80.63%, 83. 72% and 90.00% respectively. The correlation between DRR (TL) and chemotherapy efficacy was significant (Spearman rank correlation r=0.327, p=0.032). Patients with low levels of DRR in PBL presented better short-term efficacy of chemotherapy than those with high levels of DRR. Furthermore, when TM was served as an indicator, DRR medians of CR, PR, SD and PD patients were 100.00%, 81.52%, 86.05% and 95.00% respectively. There was no correlation between DRR (TM) and chemotherapy efficacy (r=0.143, p=0.361).

Discussion

Many preclinical and clinical studies have extensively investigated the association between ERCC1 expression and chemotherapy sensitivity in gastric cancer. The available dates suggest that, in gastric cancer, ERCC1 may be among the most promising predictive markers. Metzger et al. (1998) detected the ERCC1 mRNA expression in tumor tissues of 33 gastric cancer patients receiving cisplatin-based by medians of quantitative RT-PCR and found there was a significant association with response (p=0.003) and survival (p=0.034). They suggested that responding patients had low ERCC1 mRNA expression levels. Matsubara et al. (2008) studied 140 patients with advanced gastric cancer. They showed that ERCC1 expression detected by RT-PCR was significantly and inversely correlated with disease response (p=0.008) and survival (p=0.002). In protein detection, the Immunohistochemistry results of Ozkan et al. (2010) showed that gastric patients with negative ERCC1 expression experienced a long survival time than those with positive expression, when treated with platinum-based chemotherapy.

At present, the analyses of ERCC1 expression are mainly based on tumor tissue from an operative resection or gastroscopy. These bring pain to the patients and operate complicatedly. And when chemotherapy carried out, the sensitivity marker ERCC1 expression may have changed. At this time, repeated detection of ERCC1 expression using tumor tissue is inconvenient. For these reasons, clinical practices require a simpler and more convenient method of detection before individualized treatment can be realized for patients with gastric cancer.

Many studies have confirmed that PBL and tumor cells carry the homologous gene (Kaspers et al., 1991; Yang et al., 2006). Schena et al. (2012) determined DNA repair genes such as ERCC1 mRNA levels in NSCLC and HNSCC tissue, as well as PBL, from NSCLC and HNSCC patients. A statistically significant correlation (p=0.005) was found between ERCC1 mRNA expression in tumor tissue and PBL in NSCLC and HNSCC. And it could allow the introduction in clinical practice of a simple test that would measure levels of ERCC1 in PBL instead of tissue to determine prognostic and predictive factors in NSCLC and HNSCC patients. However, similar studies about gastric cancer have not been reported. Therefore, we detected ERCC1 protein expression of 53 gastric cancer patients in tumor tissue and PBL by immunohistochemical staining. We found that the ERCC1 expression in PBLs could indirectly reflect ERCC1 expression in cancer tissues.

On the other hand, because of limited samples and differences in biology behavior of different types of tumor, many studies have reached the opposite conclusions. Zhang et al. (2012) detected ERCC1 expression in PBL versus tumor tissue in gemcitabine/carboplatin-treated advanced NSCLC. They found that ERCC1 expression in

PBL and tumor tissue was negative correlated (p=0.073). Darcy et al. (2007) analysed whether platinum-DNA adducts and/or mRNA expression of the ERCC1 from PBL were associated with clinical outcome in women with epithelial ovarian cancer (EOC) treated platinum-taxane chemotherapy. They concluded that the presence of platinum-DNA adducts, but not ERCC1 mRNA expression, in PBLs was associated with better survival, but was not an independent predictor of clinical outcome in optimal advanced EOC. This shows that the formation of platinum-DNA adduct is the main cancer cell damage mechanism of platinum. ERCC1 only reflects DNA repair capacity in one stage of the complex mechanisms. Such as ERCC2, XRCC1, many molecular biomarkers can reflect the DNA repair capacity (Yin et al., 2007; Wang et al., 2012). Therefore, the efficacy of chemotherapy sometimes can not be accurately predicted by several gene detection. Slyskova et al. (2012) provided evidence on altered DRC and DNA damage levels in sporadic CRC and proposes the relevance of the NER pathway in this malignancy. Further, alterations in a complex multigene process like DNA repair should be better characterized by functional quantification of repair capacity than by quantification of individual genes transcripts or gene variants alone.

Currently, determination of the DNA repair capacity has been widely used in in tumor molecular epidemiology survey. Lou et al. (2007) found that the DNA repair capacity in PBLs of 36 lung cancer patients was significantly lower than that of non-cancer controls (p<0.05). In our research, the results of both indicators (TL and TM) showed that there was significant difference of cisplatin-induced genetic damage between gastric cancer patients and non-cancer controls. So it could be seen that decreased DNA repair capacity was one of the risk factors of gastric cancer.

In addition, response and survival of cancer patients with platinum-based chemotherapy have been predicted by DNA repair capacity of PBL. Wang et al. (2011) conducted a large and impressive study of 591 NSCLC patients treated with first-line platinum-based chemotherapy. They found that patients with NSCLC in the high tertile of DNA repair capacity (DRC) in PBLs had significantly worse overall (p=0.023) and 3-year survival (p=0.025) than those in the low tertile of DRC. It is promising to use DRC in PBLs as a prognostic factor to guide tailored individual therapeutics for patients with NSCLC. Nadin et al. (2006) showed that DNA repair capacity in PBLs of cancer patients could predict the reaction of cisplatin plus doxorubicin chemotherapy by comet assay.

It is important that comet assay (Singh et al., 1988) be a useful and convient method to evaluate DNA damage and repair capacity. The DNA repair rate (DRR), as an indicator, can represent the individual DNA repair capacity in comet assay (Zheng et al., 2005). Wei et al. (2005) detected PBL DRRs of cancer patients using bleomycin challenge test. PBLs were treated with bleomycin (20μg/mL) for 30 minutes, and then suspended for 15 minutes for DNA repair. DNA damages of pre-bleomycin and post-repair were evaluated by comet assay. Thus, we used the similar method to detect DRR in PBL of 43 patients of advanced gastric cancer, and analysed the correlation between the DRR and the efficacy of platinum-based chemotherapy. We concluded that, when TL was served as an indicator, the correlation between DRR and chemotherapy efficacy was significant. Patients with low levels of DRR in PBL presented better short-term efficacy of chemotherapy than those with high levels of DRR.

In conclusion, the ERCC1 expression in PBLs may indirectly reflect ERCC1 expression in gastric cancer tissues. Compared with non-cancer populations, patients with gastric cancer may have lower DNA repair capacity. DRR in PBL may predict the short-term efficacy for patients with advanced gastric cancer treated with platinum-based chemotherapy. However, only a few samples could be evaluated in this study. Therefore, more prospective random studies, with larger sample sizes, are needed to further analyse.

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