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Original Article

Differential gene expression pattern of the response to neoadjuvant chemotherapy in locally advanced gastric cancer

Daofu Feng[1], Wenzai Shi[2], Meiha Leong[3], Tao Li[4], Xianghui He[1], Zhixiang Zhang[1]

[1]Department of General Surgery, Tianjin Medical University General Hospital, Tianjin 300052, China; [2]Department of Hepatobiliary Surgery, Peking University International Hospital, Beijing 102206, China; [3]Department of Surgery, Kiang Wu Hospital, Macao, China; [4]Department of General Surgery, Chinese PLA General Hospital, Beijing 100028, China

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Abstract: To improve the survival of gastric cancer, neoadjuvant chemotherapy followed by surgery has been a promising treatment protocol. However, non-responders have to suffer from adverse effects and miss the opportunity of other possible treatments. To elucidate the molecular basis of the response to neoadjuvant chemotherapy, the gene expression pattern was analyzed by a microarray-based method. Based on our previous retrospective study, we examined the gene expression of twelve patients who underwent S-1 plus oxaliplatin (SOX) neoadjuvant chemotherapy before curative surgery (R0) for stage III gastric cancer. Three tumor tissues without neoadjuvant chemotherapy were collected as the control group. These three groups were compared on functional and pathway enrichment analysis. The results were mainly shown that differentially expressed genes in responders and non-responders were highly enriched for genes involved in cytokine-cytokine receptor interaction and NK cell mediated cytotoxicity. Considering the drug sensitivity of oxaliplatin and S-1, microarray analysis significantly demonstrated 3 up-regulated genes and 1 down-regulated gene in DNA damage repair pathway which may play an important role in drug resistance (responders vs. non-responders). Thus, microarray analysis can efficiently evaluate the gene expression after neoadjuvant chemotherapy for gastric cancer, which may better understand tumor chemosensitivity.

Keywords: Gastric cancer, neoadjuvant chemotherapy, chemosensitivity, gene expression profiling, immune response

Introduction

Gastric cancer (GC) is a common malignancy and the second leading cause of all cancer-related deaths worldwide [1]. The prevalence is particularly high in East Asia, such as Japan, South Korea and China [2]. The mainstay of treatment for locally advanced GC is curative surgery combined with neoadjuvant or adjuvant chemotherapy in Asia. Numerous chemotherapeutic regimens have shown that this comprehensive treatment strategy is apparently effective against GC [3-6]. Doublet chemotherapy using S-1 and cisplatin (SP) has been used as neoadjuvant chemotherapy for stage III gastric cancer in Japan [7, 8]. However, cisplatin is more toxic than oxaliplatin and SP regimen is not superior to S-1 plus oxaliplatin (SOX) with regard to relapse-free survival [9, 10]. Our previous study showed that SOX neoadjuvant chemotherapy could be used for locally advanced gastric cancer effectively and safely [11]. Despite the therapeutic advances which have improved overall survival of GC patients, the beneficial effects from neoadjuvant chemotherapy can hardly be predicted by clinical parameters. Non-responders often lose their precious time to receive more sensitive treatments [12]. Therefore, it is essential to establish a reliable method that can identify molecular factors on the response to neoadjuvant chemotherapy.

GC biological characteristics are one of the most significant factors that affect chemosensitivity. Molecular analyses (Polymerase Chain Reaction and immunehistochemistry) for tumor

Table 1. Characteristics of 15 patients with gastric cancer in responders, non-responders and non-chemotherapy tissues

ID	Gender	Age (yr)	Tumor size (mm)	cTNM	pTNM	LNM	Response
A2346-2	F	45	15	T4bN+M0	T2N0M0	0/40	R
A2347-2	M	51	14	T4bN+M0	T2N0M0	0/22	R
A2348-2	M	68	26	T4aN+M0	T1N0M0	0/32	R
A2351-2	M	57	50	T4aN+M0	T2N0M0	0/49	R
A1934	M	72	23	T4aN+M0	T2N0M0	0/31	R
A2349-2	M	65	50	T4bN+M0	T2N0M0	0/29	R
A4125	M	63	20	T4aN+M0	T4aN1M0	1/57	NR
A4127	M	48	15	T3N+M0	T3N1M0	1/19	NR
A4130	M	42	80	T4aN+M0	T4aN3M0	20/33	NR
A4131	M	53	50	T3N+M0	T3N3M0	21/74	NR
A2875	M	60	30	T4aN+M0	T4aN1M0	2/23	NR
A2876	M	59	50	T3N+M0	T3N1M0	1/24	NR
A1953	M	69	55	T4aN+M0	T4aN1M0	2/42	NC
A1954	F	60	55	T4aN+M0	T4aN2M0	3/20	NC
A4133	M	45	65	T4aN+M0	T4aN3M0	34/57	NC

Clinical diagnosis of tumor depth, lymph node metastasis and distant metastasis before neoadjuvant chemotherapy were classified based on TNM classification (7th edition). The ratio under lymph node metastasis represented the number of affected nodes over the number of nodes evaluated based on pathological results. cTNM, clinical TNM; pTNM, pathological TNM; LNM, lymph node metastasis; R, responders; NR, non-responders; NC, non-chemotherapy tissues.

tissues after neoadjuvant chemotherapy have been conducted to clarify the biological characteristics of GC [13, 14]. However, in these methods, only a few of genes have been addressed in these studies. Large amounts of genetic alternations are associated with the development and progression of GC, and their variations may affect multiple gene expression [15]. Furthermore, many molecular pathways may relate to the sensitivity of GC to chemotherapy. Microarray gene expression profiling can simultaneously assess the expression of thousands of genes, which was regarded as the powerful method for clarifying the biological characteristics of GC. The integrative approach has been used to identify the genes that could serve as novel biomarkers to predict recurrence in GC after curative resection [16]. Recent study also showed that gene expression profiling using surgically resected samples can assess the chemosensitivity after postoperative chemotherapy [17]. Accordingly, we further explored the differential gene expression, functions and pathways after neoadjuvant chemotherapy in locally advanced GC by gene expression profiling.

In this study, we mainly focused on molecular understanding of gene expression on the response to neoadjuvant chemotherapy acquired by employing a systematic approach. Using surgically resected samples, responders after neoadjuvant chemotherapy were compared with non-responders and patients without chemotherapy. As for SOX regimen, the drug resistance of S-1 and oxaliplatin was mainly associated with folate metabolism and DNA damage repair respectively. Hundreds of differential genes were detected in various functional pathways by the integrative approach. In addition, several differential genes had been highly expressed with the response to neoadjuvant chemotherapy in DNA repair mechanism, which may be associated with tumor chemoresistance in GC.

Methods and materials

Patients and samples

All patients clinically diagnosed as locally advanced gastric cancer (T3/T4, N+, M0) and eligible for neoadjuvant chemotherapy were enrolled into the study. The eligibility and exclusion criteria were based on our previous study [11]. Four cycles of neoadjuvant chemotherapy were performed. Tissue samples were collected from patients who underwent gastrectomy from 2012 to 2013. Based on the guideline of Response Evaluation Criteria in Solid Tumors (RECIST) criteria 1.1 [18] and pathological results, six tissue samples were selected as responders (partial response) and six other ones were evaluated as non-responders (stable disease or progressive disease) in the study. Three tumor tissues without neoadjuvant chemotherapy were collected as the control group. These 15 samples were retrospectively selected from our previous study [11]. The samples were stored at -80°C Written informed consent was obtained from all patients. The study was approved by the Human Research

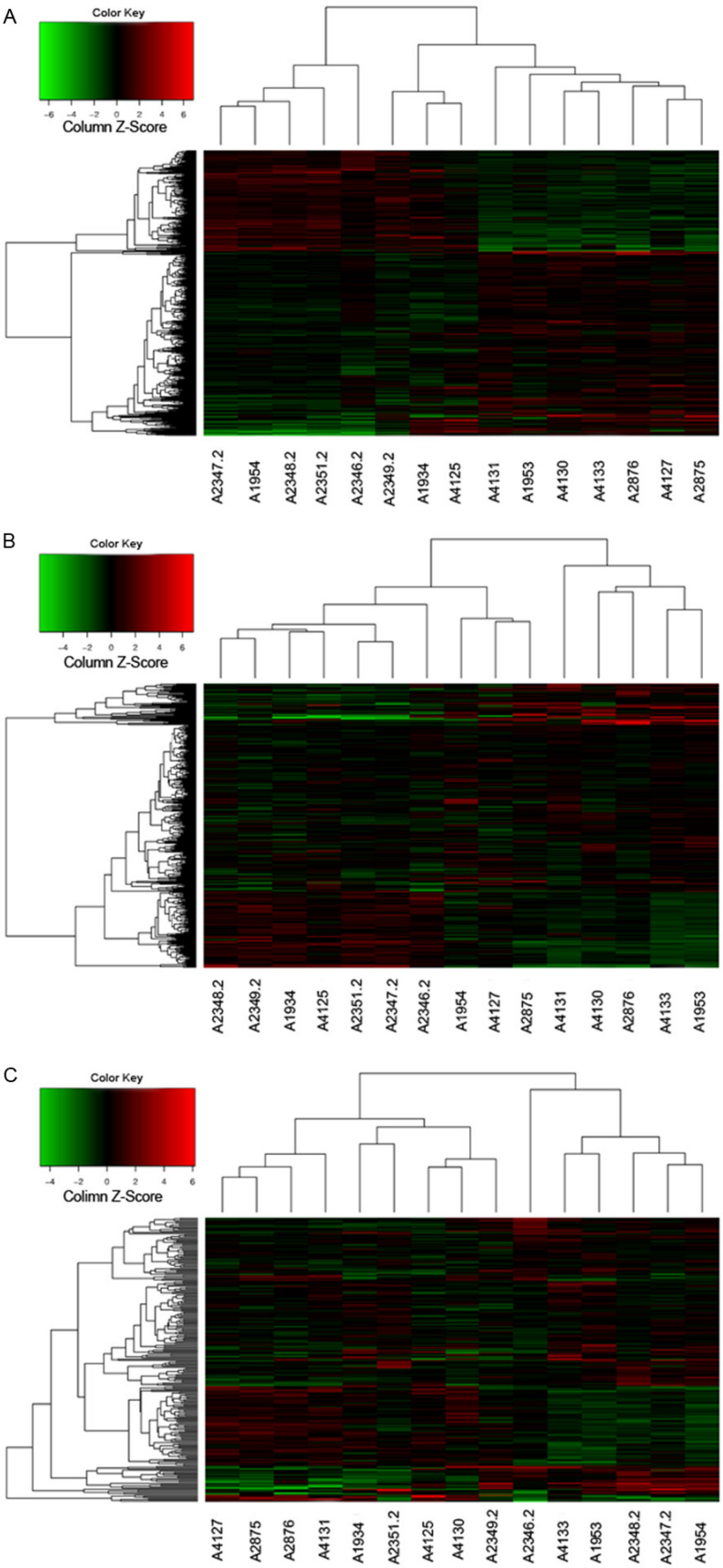

Figure 1. Hierarchical clustering. A. Differentially expressed genes in responders and non-responders were used in the hierarchical clustering analysis. Red denotes high expression levels, whereas green denotes low expression levels. B. Differentially expressed genes in responders and non-chemotherapy tissues were used in the hierarchical clustering analysis. Red denotes high expression levels, whereas green denotes low expression levels. C. Differentially expressed genes in non-responders and non-chemotherapy tissues were used in the hierarchical clustering analysis. Red denotes high expression levels, whereas green denotes low expression levels.

Ethics Committee of Tianjin Medical University General Hospital. According to the TNM classification [19], the clinical and pathological features of the patients were listed in **Table 1**.

RNA extraction

Total RNAs were extracted from gastric samples without lymph nodes from locally advanced GC patients using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Then, the RNA was purified by the RNeasy mini kit (Qiagen, Valencia, CA, United States). RNA quality was assessed for each sample using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, United States). The RNA concentration was measured by Nanodrop 2000 (Thermo Scientific, Wilmington, DE, United States). The RNAs were accepted for further analysis by A260/A280 ratios of 1.7-2.2 in the Nanodrop analyses and 28S/18S ribosomal peak

Table 2. The GO biological process and molecular function associated with differentially expressed genes in responders versus non-responders

GO categories	GO-ID	*P* values	m[a]	n[b]	Description
Biological process	0007165	3.29E-12	1634	69	Signal transduction
	0007275	1.11E-11	1049	52	Multicellular organismal development
	0043283	1.37E-10	1684	66	Biopolymer metabolic process
	0006139	2.85E-9	1244	52	Nucnucleic acid metabolic process
	0048856	2.85E-9	1013	46	Anatomical structure development
	0048513	4.26E-9	571	33	Organ development
	0048731	2.06E-8	861	40	System development
	0044260	2.06E-8	1131	47	Cellular macromolecule metabolic process
	0016070	2.85E-8	841	39	RNA metabolic process
	0019538	2.85E-8	1231	49	Protein metabolic process
Molecular function	0004872	5.6E-6	583	29	Receptor activity
	0004888	1.47E-5	418	23	Transmembrane receptor activity
	0022892	6E-4	392	19	Substrate specific transporter activitvity
	0016462	6E-4	226	14	Pyrophosphatase activity
	0016817	6E-4	228	14	Hydrolase activity on acid anhynhydrides
	0022891	9.83E-4	344	17	Transmembrane transansporter activity
	0030169	1.11E-3	12	4	Low density lipoprotein binding
	0005509	1.11E-3	104	9	Calcium ion binding
	0051082	1.38E-3	42	6	Unfolded protein binding
	0022857	1.7E-3	375	17	Transmembrane transporter activity

[a]The number of genes in the reference set annotated to a certain GO term. [b]The number of the input genes annotated to a certain GO term.

ratios of >0.7 with RIN (RNA Integrity Number) ≥7.0 in the Bioanalyzer.

Microarray procedures and quality control

Gene expression of 15 surgically resected samples was analyzed in three groups by Affymetrix Gene Chip Prime View Human Gene Expression Array (Santa Clara, CA, United States). The Gene Chip 3' IVT Express Kit was used to acquire amplified RNA with further purification, and hybridization was performed. The signals were scanned by Gene Chip Scanner 3000. Quality control of gene chips was measured by Signal Histogram, Relative Signal Box Plot, Pearson's Correlation and Principal Component Analysis (PCA). The volcano plot and scatter diagram were described for the selection of differentially expressed genes.

Hierarchical clustering

Hierarchical cluster analysis was performed by using Gene Math 2.0 software (Applied Maths, Inc., Austin, TX). The up- and down-regulated genes were compared among the responders, non-responders and non-chemotherapy tissues. We transformed the expression of all our tissue samples into standard scores, and performed hierarchical clustering for three groups.

Functional and pathway enrichment analysis

Over- and under-expressed genes were classified by Gene Ontology (GO) category [20]. GO includes three parts: molecular function, biological process and cellular component according to the key functional classification of the National Center for Biotechnology Information (NCBI). Based on chemosensitive mechanism of SOX regimen, DNA damage repair and folate metabolism were analyzed with gene chip data as follows. To identify the biomarkers associated with chemoresistance, differentially expressed genes were annotated by the Gene Ontology file (GO: 0006281, the process of restoring DNA after damage) and the other ontology file (GO: 0006766, the chemical reactions and pathways involving folate metabolism). Pathway annotations of the differentially expressed genes were summarized from KEGG [21].

Table 3. The GO biological process and molecular function associated with differentially expressed genes in responders versus tissues without chemotherapy

GO categories	GO-ID	*P* values	m[a]	n[b]	Description
Biological process	0007165	1.76E-6	1634	39	Signal transduction
	0043283	4.37E-5	1684	36	Biopolymer metabolic process
	0044267	4.37E-5	1117	28	Cellular protein metabolic process
	0044260	4.37E-5	1131	28	Cellular macromolecule metabolism
	0019538	5.85E-5	1231	29	Protein metabolic process
	0016310	2.5E-4	313	13	Phosphorylation
	0043687	2.5E-4	476	16	Post translational protein modificatiotion
	0006468	3.52E-4	279	12	Protein amino acid phosphorylation
	0006457	6.92E-4	58	6	Protein folding
	0007242	8.57E-4	667	18	Intracellular signaling cascade
Molecular process	0016772	9.39E-4	424	15	Phoshosphorustransferase activity
	0016301	1.88E-3	369	13	Kinase activity
	0030234	1.88E-3	323	12	Enzyme regulator activity
	0016773	1.88E-3	334	12	Phosphorus transferase activity
	0004672	1.88E-3	285	11	Protein kinase activity
	0004674	3.21E-3	205	9	Catalysis of ATP reaction
	0005102	1.59E-2	377	11	Receptor binding

[a]The number of genes in the reference set annotated to a certain GO term. [b]The number of the input genes annotated to a certain GO term.

Statistical analysis

Microsoft Excel 2010 was performed to collect the data (Supplemental Materials). The SPSS 19.0 software was used to analyze the data with Fisher, ANONA (analysis of variance) and chi-square tests in different issues: the samples of 6 responders, 6 non-responders and 3 non-chemotherapy samples were mainly compared by multiple comparisons using ANONA and chi-square test; Fisher's test and chi-square test were applied to classify the GO category. The false discovery rate (FDR) cut-off was 0.05. The differentially expressed genes among response, non-response and non-chemotherapy tissues were filtered by Fold Change >1.5 and *P* value <0.05. *P*-values below 0.05 were regarded as statistical significance.

Results

Genome-wide expression analysis

We identified differentially expressed genes (up- and down-regulated genes) from 6 responders, 6 non-responders and 3 tumor tissues without chemotherapy. The *P*-value was less than 0.05 in t-tests, and the fold change was more than 1.5 for selection. To validate the relationships among responders, non-responders and non-chemotherapy group, we performed hierarchical clustering analyses using the gene expression data. Comparing responders and non-responders, we identified 458 up-regulated genes and 241 down-regulated genes (**Figure 1A**). When responders and non-chemotherapy tissues were compared, 108 differentially expressed genes were up-regulated and 283 genes were down-regulated (**Figure 1B**). With the comparison of non-responders and non-chemotherapy tissues, 172 up-regulated genes and 85 down-regulated genes were shown (**Figure 1C**). The differentially expressed genes were then fed into function and pathway analyses to profoundly understand the molecular basis of SOX neoadjuvant chemotherapy.

Functional enrichment analysis

The gene expression in neoadjuvant chemotherapy was analyzed to explore the molecular function and biological processes in GO. Based on the microarray data between the responders and non-responders, GO analyses indicated that 10 top GO terms were focused on signal transduction, multicellular organismal development, biopolymer metabolic process, anatomical structure development, organ development,

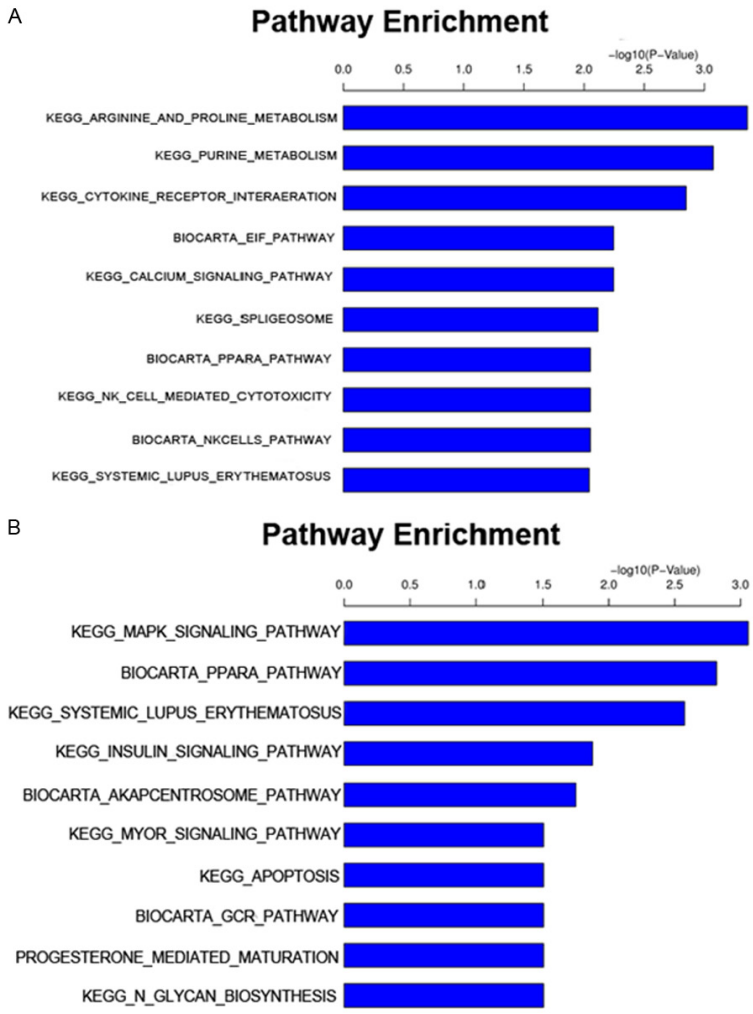

Figure 2. Pathway analysis of differentially expressed genes according to the KEGG database. A. Top-ranking pathways between responders and non-responders identified by KEGG. B. Top-ranking pathways between responders and non-chemotherapy tissues identified by KEGG.

system development, cellular macromolecule metabolic process, RNA metabolic process and protein metabolic process in biological processes (**Table 2**). The main GO categories for molecular function were receptor activity, transmembrane receptor activity, substrate specific transporter activity, pyrophosphatase activity, low density lipoprotein binding, calcium ion binding, unfolded protein binding and transmembrane transporter activity (**Table 2**). According to the differential genes and functions, the diversity in these categories between responders and non-responders suggested that the chemosensitivity of SOX regimen may be associated with receptor interactions, signaling pathways, cell metabolism processes and transmembrane transport.

As for responders and tissues without chemotherapy, the GO terms were mainly signal transduction, biopolymer metabolic process, cellular protein metabolic process and cellular macromolecule metabolic process in biological processes (**Table 3**). In addition, the GO categories for molecular function were enzyme regulator activity, kinase activity, protein kinase activity and receptor binding. The result indicated that the diversity may be induced by the sensitivity of tumor tissues in neoadjuvant chemotherapy and the pharmacological effects of chemotherapy.

Non-responders were also compared with the non-chemotherapy group to identify the pharmacological effects induced by chemotherapy. However, no data were shown in biological process and molecular function in the GO category.

Pathway enrichment analysis

Pathway analyses were used to identify the KEGG biological pathways associated with differentially expressed genes. The genes in responders and non-responders were strongly associated with cytokine-cytokine receptor interaction, NK cell mediated cytotoxicity and calcium signaling pathway (**Figure 2A**). In addition, receptor interaction and signaling pathway were highlighted. Therefore, the pathway profiling was consistent with the results of the GO categories in biological functions. In contrast, the genes between responders and non-chemotherapy tissues were associated with MAPK signaling pathway, apoptosis, NK cell mediated cytotoxicity and toll-like receptor signaling pathway (**Figure 2B**).

Potential biomarkers for SOX chemosensitivity

The DNA damage repair and folate metabolism were intrinsically related to the drug resistance of metabolic mechanism in oxaliplatin and S-1,

Table 4. Differentially expressed biomarkers associated with the DNA damage repair in responders, non-responders and non-chemotherapy tissues

Multiple comparison	Gene Symbol	Regulation	Fold change	Log Fold change	*P* values
R vs. NR	HUS1	Up	1.6677043	0.73786354	0.010914844
	RECQL5	Down	-1.7384835	-0.7978294	0.001071271
	XRCC4	Up	1.7419167	0.80067563	0.016486978
	GADD45G	Down	-2.12068	-1.084527	8.12407E-05
R vs. NC	BTG2	Down	-2.082077	-1.0580235	0.041781284
	GADD45G	Up	2.0418868	1.0299028	0.004517132
NR vs. NC	DDB2	Down	-1.5816944	-0.6614709	0.011229033

R, responders; NR, non-responders; NC, non-chemotherapy tissues.

respectively. A variety of DNA repair pathways include direct reversal, base excision repair, nucleotide excision repair, bypass, double-strand break repair pathway, and mismatch repair pathway. In responders and non-responders, we identified that HUS1 (FC=1.67, P=0.011), RECQL5 (FC=-1.74, P=0.001) and XRCC4 (FC=1.74, P=0.016) may be associated with tumor resistance (**Table 4**). Furthermore, GADD45G was down-regulated in responders versus non-responders, while GADD45G was up-regulated in responders versus non-chemotherapy tissues. BTG2 (FC=-2.08, P=0.04) was only down-regulated when responders were compared with tumor tissues without chemotherapy. DDB2 in the non-responders versus the non-chemotherapy group was down-regulated (**Table 4**). For folate metabolism, our study could not find any differentially expressed gene among three groups.

Discussion

In recent decades, neoadjuvant chemotherapy has been accepted as the standard treatment of choice for locally advanced gastric cancer. Several studies have highlighted the persuasive evidence that preoperative chemotherapy is superior to postoperative multimodal therapy [4, 5], even though the response of gastric carcinomas to chemotherapy varies between complete remission and tumor resistance. In these studies, some patients were not beneficial from the preoperative treatment due to the drug resistance which could be associated with DNA damage repair and folate metabolism. For this reason, it will be the clinical significance that responders and non-responders can be distinguished by specific gene expression before providing the best multimodal support to surgical resections [22].

Microarray gene expression analyses for gastric cancer have been reported to identify diagnostic biomarkers [23] and to reveal differentially expressed genes associated with prognosis [24]. However, it has not been used to identify the gene expression patterns associated with chemoresistance of SOX regimen. We analyzed the microarray data gained from responders, non-responders and the tumor tissues without SOX neoadjuvant chemotherapy in 15 locally advanced GC patients. The gene expression data were subjected to hierarchical clustering, GO categories and pathway analyses.

Few studies have reported the relationships between S-1 plus oxaliplatin for gastric cancer and over-expressed genes. Tetsuya et al [17] indentified 5 specific miRNAs associated with gastric cancer recurrence after S-1 adjuvant chemotherapy. As for colon cancer, Nakajima et al [25] found that two miRNAs were strongly related to S-1 plus cisplatin chemotherapy. Nearly 70% patients underwent S-1 chemotherapy in their study. Our previous study showed that SOX neoadjuvant chemotherapy was effective and safe for stage II and III gastric cancer. However, a few of patients were not beneficial from the treatment because the tumors were not shrunk after neoadjuvant chemotherapy [11]. Thus, it was essential for locally advanced gastric cancer to find out the chemosensitivity of SOX regimen by an integrative systematic approach.

Based on our functional and pathway analyses, the differentially expressed genes in responders and non-responders were highly enriched for genes involved in cytokine-cytokine receptor interaction and NK cell mediated cytotoxicity. The implication is closely related to immune reaction for the response to SOX regimen. In

the comparison of responders and non-chemotherapy tissues, the genes were associated with NK cell mediated cytotoxicity and toll-like receptor signaling pathway. To rule out the pharmacological effect of chemotherapy, we also compared the non-responders and non-chemotherapy groups, while there was no differentially expressed gene in GO categories. Thus, the SOX chemosensitivity may be involved in immune reactions.

Furthermore, we examined the DNA damage repair pathways and folate metabolism process by GO categories, which had previously been reported to be mainly associated with the resistance of platinating and 5-FU based agents [26-28]. ERCC1 has been recognized as an independent prognostic marker in DNA damage repair pathways, which pointed out that higher expression was associated with a poorer prognosis [29]. In our study, four differentially expressed genes were found in responders and non-responders. The protein encoded by HUS1 is able to form a Rad9-Rad1-Hus1 complex involved in cell cycle arrest in response to DNA damage. Shikawa et al reported that lower expressed HUS1 was related to higher malignancy in gastric cancer [30]. The result was consistent with up-regulated HUS1 in responder tissues. RECQL5 is one of five RecQ helicases to maintain the genome stability and participate in mismatch repair process. Futami et al reported that chemosensitivity of camptothecin can be increased by down regulation of RECQL5 [31]. Our study also suggested that RECQL5 played an important role in biopolymer metabolic process and nucnucleic acid metabolic process by GO analysis (**Table 2**). XRCC4 is a major repair gene for DNA double-strand breaks in the non-homologous end joining pathway. The association of XRCC4 with neoadjuvant chemotherapy of gastric cancer has never been reported. Thus, further investigation need to be performed to profoundly identify the gene function for chemothrapy. GADD45G is an important member of GADD45 family identified as a growth arrest, which could serve as a functional tumor suppessor gene and a therapeutic target. The study of Ying et al provided strong evidence that GADD45G expression is frequently reduced or silenced in multiple tumors [32]. GADD45G expression in our study may be influenced by the efficiency of therapeutic agents. BTG2 is involved in many biological activities in cancer cells acting as a tumor suppressor. However, the result revealed that BTG2 was down-regulated in responders, which may be induced by pharmacological effects. In addition, BTG2 can be regulated by many factors involving different signal pathways [33]. Thus, the effect of BTG2 in SOX regimen will be further investigated. DDB2 closely associated with P53 signaling pathway participates in nucleotide excision repair. DDB2 was up-regulated in non-responders which indicated that chemotherapy led to DNA damage and activated DNA repair. In contrast, non-responsive tumors perform genetic alterations in the DNA damage response pathways, allowing them to escape apoptosis on chemotherapy.

Meanwhile, folate metabolism was also evaluated by GO analysis. The recent studies concluded that dihydropyrimidine dehydrogenase (DPD) expression was associated with enhanced benefit from adjuvant S-1 in gastric cancer [34, 35]. However, we could not find any differentially expressed gene in folate metabolism after SOX neoadjuvant chemotherapy, which was consistent with our previous study [36]. Thus, the mechanism of doublet chemotherapy may be more complicated than single chemotherapy.

The limitation of our study is the relatively small number of samples analyzed. Because the tissues after neoadjuvant chemotherapy may not be correctly evaluated only by endoscopic biopsy, all tissues were taken in the postoperative setting. The pre-treatment samples from the patients were not assessed in this study. Thus, our results need to be proved by further investigation. Future attention should also focus on the genes which have yielded the promising results.

We have analyzed the differentially expressed genes in SOX neoadjuvant chemotherapy for locally advanced gastric cancer by using an integrative systematic approach. The study showed that surgically resected samples could assess the tumors according to their historical response to chemotherapy. We also found that genes highly expressed in responders were involved in immune-related signaling events. The encouraging results warrant validation of the potential genes with large amount of patients and prospective trials in future.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhixiang Zhang, Department of General Surgery, Tianjin Medical University General Hospital, No. 154 Anshan Road, Heping District, Tianjin 300052, China. E-mail: zhangzhixiang63@sina.com

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