

Long noncoding RNA XIST is a prognostic factor in colorectal cancer and inhibits 5-fluorouracil-induced cell cytotoxicity through promoting thymidylate synthase expression

Yang Xiao¹, Usenko Alexander Yurievich¹ and Smorzhevskiy Valentyn Yosypovych¹

¹Department of Surgery and Transplantology, P. L. Shupyk National Medical Academy of Postgraduate Education, Kyiv, Ukraine

Correspondence to: Yang Xiao, email: xiaoyangbj1984@163.com

Keywords: lncRNA XIST, colorectal cancer, 5-fluorouracil, chemoresistance, thymidylate synthase

Received: May 13, 2017

Accepted: July 25, 2017

Published: August 24, 2017

Copyright: Xiao et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License 3.0 (CC BY 3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ABSTRACT

A major reason for the failure of advanced colorectal cancer (CRC) treatment is the occurrence of chemoresistance to 5-fluorouracil (5FU)-based treatment. Recent studies have shown that long non-coding RNAs (lncRNAs) are critical regulators in chemoresistance. By using the next generation HiSeq sequencing assay, we identified lncRNAs showing differential expression levels in 5FU resistant and non-resistant CRC patients. RT-qPCR was then performed for validation in tissues and serum samples, and lncRNA XIST was verified to be up-regulated in non-responding patients and have considerable diagnostic potential to identify responding patients from non-responding patients. In addition, increased serum XIST level was associated with poor response and lower survival rate in CRC patients receiving 5FU-based treatment. Subsequently, the 5FU resistant (5FU-R) cell lines were established, and lncRNA XIST was significantly up-regulated HT29 5FU-R and HCT116 5FU-R cells. Furthermore, knockdown of XIST reversed 5FU resistance while enhanced XIST could restrained the 5FU-induced cell cytotoxicity in both CRC cell lines. Western blotting and immunofluorescence analysis indicated that XIST promoted the expression of thymidylate synthase, a critical 5FU-targeted enzyme. In conclusion, our integrated approach demonstrates that increased expression of lncRNA XIST3 in CRC confers a potent poor therapeutic efficacy, and that lncRNA XIST participated in 5FU resistance through promoting the expression of thymidylate synthase. Thus, specific silence of lncRNA XIST could be a future direction to develop a novel therapeutic strategy to overcome 5FU resistance of CRC patients.

INTRODUCTION

Colorectal cancer (CRC) is among the leading causes of mortality and morbidity throughout the world, thus representing a major public health problem. It is the second- and third-most commonly diagnosed cancer in females and males, respectively [1, 2]. Metastasis is the major cause of death for patients with CRC and increases the risk of tumor recurrence [3]. Fluoropyrimidine is an antimetabolite used as the first-line chemotherapeutic agent of various cancers. However, the response rates of 5FU for advanced CRC is only

10–15%[4]. Fluoropyrimidine-based chemotherapy (e.g., 5-fluorouracil (5FU)) has been the cornerstone of treating advanced CRC for over a half century. However, nearly all patients receiving chemotherapy finally develop drug resistance, and this has been a major obstacle to improving the efficiency of colorectal cancer treatment [5]. Thus, revealing the underlying mechanism and finding new therapeutic approaches are necessary for developing effective therapies for colorectal cancer patients.

Chemotherapy is a systemic treatment that destroys reproducing cells, but it cannot differentiate between normal and cancerous cells. Side effects occur when

normal cells become damaged. Moreover, resistance to chemotherapy has been more and more common, mostly due to the drug accumulation [6] or enhancement of DNA repair [7] and activation of detoxification [8, 9]. A major reason for CRC chemoresistance is to resist drug-induced cell death through disruptions of apoptotic pathways [10]. It is widely accepted that 5FU exerts its anti-tumor effect mainly by targeting a series of enzymes including thymidylate synthase (TS). Thus, TS participates in the regulation of cell proliferation process and can be inhibited by 5FU treatment, especially for patients receiving standard 5FU based chemotherapy regimens.

During the past decade, large-scale sequencing efforts and the ENCODE project have revealed that a large

fraction of the human noncoding genome is transcribed [11]. Noncoding RNAs (ncRNA) are found throughout the genome. The function and clinical significance of short ncRNAs, such as miRNA and siRNA were elucidated first and then, long noncoding RNAs (lncRNAs) were reported more recently. Among more than 3000 human lncRNAs, less than 1% have been functionally characterized, but some of these are involved in normal development and in diseases, including cancer [12]. Recent extensive annotation of lncRNAs has been performed in multiple model organisms, revealing that they are often expressed in a spatial- and temporal-specific pattern. Moreover, a large amount of lncRNAs has been recognized as promising biomarkers for diagnosis and prognosis as well as critical

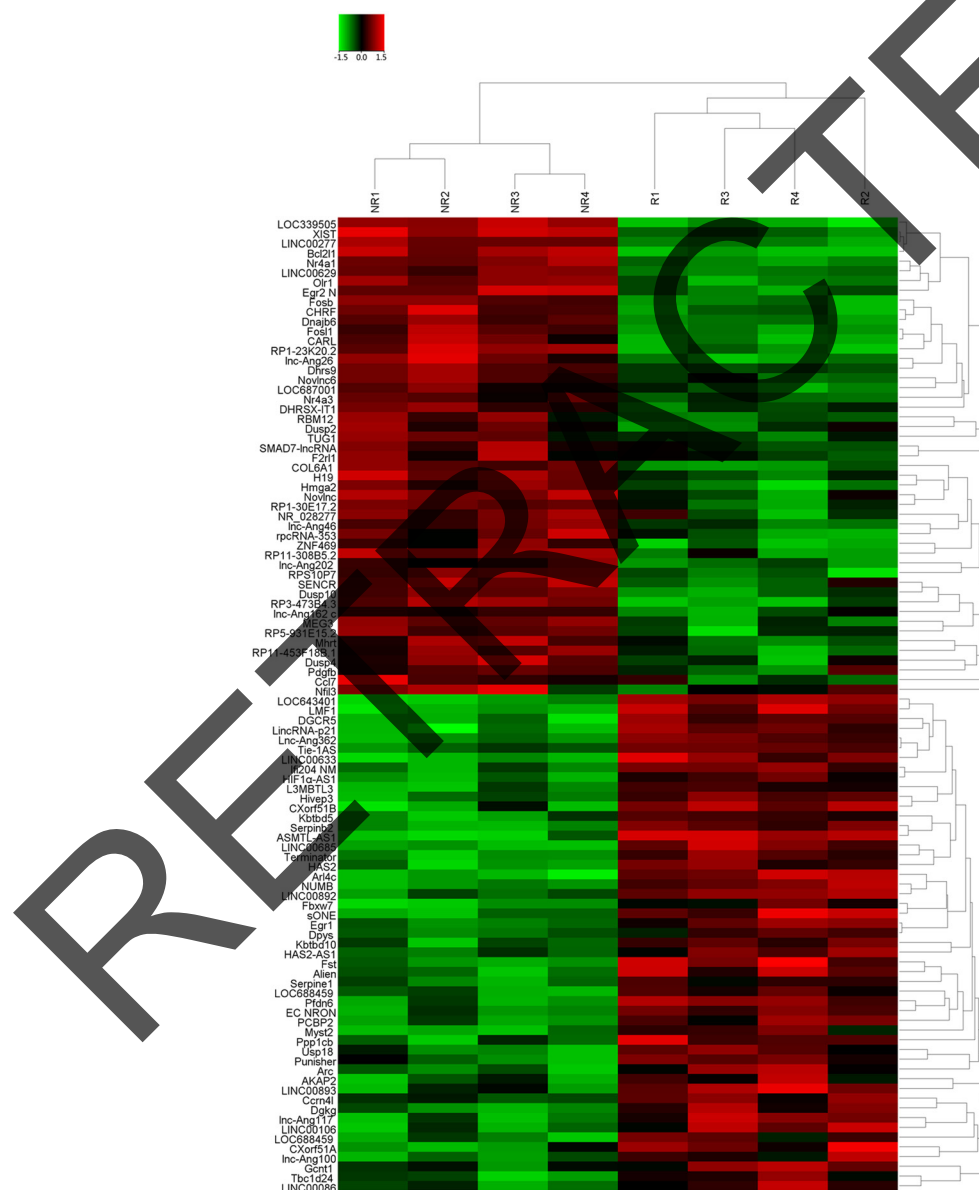


Figure 1: The heat map shows expression of the 100 lncRNAs most up- or down-regulated in CRC responding compared with non-responding patients to 5FU treatment. The top 50 lncRNAs up- and down-regulated in non-responding group are shown in the top and bottom halves, respectively. The heat map was generated with an R package using normalization across rows (tissues).

Table 1: Candidate lncRNAs selected on a basis of the Hiseq analysis

Seq name	Location	Regulation (NR vs R)	Fold change	P value
LOC339505	Chr1p36.12	Up	93.2865913	0.00002894
XIST	ChrXq13.2	Up	63.9830265	0.00301645
LINC00277	Chr15q23	Up	28.3927551	0.00718443
LOC643401	Chr5p14.1	Down	52.4439156	0.00054537
LMF1	Chr16p13.3	Down	33.6422393	0.00176431
DGCR5	Chr22q11.21	Down	25.9623587	0.01439265

NR: non-response; R: response.

regulators of pathways involved in cancer progression including NSCLC, gastric cancer and CRC [13-16]. However, the association of specific lncRNA expression with 5FU resistance in CRC is not well known.

In the current study, a high-throughput Hiseq sequencing was firstly performed to find the potential aberrant lncRNAs. Then, the reverse transcription quantitative real-time PCR (RT-qPCR) assays were used to validate the upregulation or downregulation of those lncRNAs in both primary tissues and serums from CRC patients receiving 5FU treatment, and these lncRNAs may directly participate in the development of chemoresistant process in CRC. The results indicated that the enhanced lncRNA XIST expression was correlated with clinical outcome of 5FU based treatment. Additionally, XIST restrained the 5FU- induced cell cytotoxicity through EZH2.

RESULTS

Discovery of potential lncRNAs by Hiseq sequencing method

Four tissue samples pooled from CRC patients showing response and four from patients showing no response to 5FU treatment was subjected to Hiseq sequencing. On the basis of the data obtained from Hiseq sequencing, we then identified 677 lncRNAs that were differently expressed more than 2-fold change. Subsequently, we narrowed the scope of the study to the 100 most aberrant expressed RNAs, including 50 up-regulated lncRNAs and 50 down-regulated lncRNAs (shown in the heat map, Figure 1). These lncRNAs should be easily detected with a Ct value less than 35, easily designed primers and have stable expression in both primary tissues and serum samples. According those definition, we finally restricted to six lncRNAs from three of up-regulated group and three of down-regulated group (Table 1). In addition, Another four lncRNAs were brought into our study, as they were reported to have potential function in the development of 5FU resistance in CRC [17-20]. Thus, we discovered 10 candidate lncRNAs

which may be potential biomarkers for CRC patients receiving 5FU treatment, pending further validation.

lncRNA XIST is up-regulated in CRC patients showing non-response to 5FU treatment

RT-qPCR was performed to further test the 10 lncRNAs selected through the Hiseq sequencing method by using 70 primary CRC tissues from patients showing objective response to treatment and 70 from patients showing no response. Among these, four lncRNAs (XIST, UCA1, CCAL, DGCR5) were found significantly dysregulated in responding tissues compared with non-responding tissues (Table 2). Take a step further, lncRNA XIST, UCA1, CCAL and DGCR5 were further validated in an another group of 120 of serum specimens from 60 CRC patients showing response and 60 showing no response. Among the four candidate lncRNAs, only lncRNA XIST was significantly desregulated with a dramatically elevated expression in patients that showed no response compared to patients that showed response to 5FU (Figure 2). Thus, we focus on the role of XIST in 5FU chemoresistance.

Increased expression of serum XIST indicates poor response to 5FU treatment in CRC patients

After having validated the up-regulation of lncRNA XIST in non-responding CRC patients, we further investigated if XIST had clinical predictive value for 5FU treatment among CRC patients. Thus, receiver operator characteristic (ROC) curve analysis was firstly performed to evaluate the potential diagnostic value of XIST in patients receiving 5FU based therapy. Our data showed that the area under the ROC curve (AUC), diagnostic sensitivity and specificity were 0.756, 71.7% and 68.3%, respectively (Figure 3A). we then divided these patients into high (63 patients) and low (57 patients) XIST level groups on the basis of the cut-off value (1.38) obtained from the ROC curve. In high XIST level group, the responding patients occupies 31.75% of all, while this proportion in low XIST level group was 70.15%, which is significantly higher than that in high XIST

Table 2: Expression of 10 candidate lncRNAs in CRC patients showing response or non-response to 5FU treatment [median (interquartile range)]

LncRNA	Response	Non-response	P value
LOC339505	1.59 (0.47-2.32)	1.86 (1.15-2.67)	0.28
XIST	0.97 (0.41-1.95)	1.69 (0.49-2.44)	<0.01
LINC00277	1.08 (0.33-2.14)	1.27 (0.50-2.99)	0.17
UCA1	0.87 (0.21-1.78)	1.51 (0.32-2.08)	<0.05
PANDAR	1.46 (0.40-2.07)	1.65 (0.64-2.87)	0.57
MALAT1	1.07 (0.31-2.23)	1.37 (0.29-2.03)	0.21
CCAL	1.36 (0.40-2.39)	1.94 (0.35-2.79)	<0.05
LOC643401	1.35 (0.26-2.09)	1.02 (0.30-1.88)	0.07
LMF1	1.22 (0.59-2.20)	0.98 (0.33-1.76)	0.15
DGCR5	1.48 (0.46-2.31)	0.93 (0.27-1.99)	<0.01

level group (Figure 3B). Finally, Kaplan-Meier survival analysis further suggested that patients showing high XIST expression level indicated a relatively poor overall survival rate and recurrence-free survival rate ($P=0.002$ and 0.014 , respectively. Figure 3C and 3D). Furthermore, we performed Cox regression univariate/multivariate analysis to identify whether XIST or other clinical parameter is an independent indicator for overall survival of CRC patients who received 5FU based therapy. The results indicated that serum XIST level, TNM stage and distant metastasis were identified as potential independent prognostic factors for overall survival of CRC patients receiving 5FU treatment (Table 3).

XIST is up-regulated in 5FU-resistant CRC cell lines

After having uncovered the clinical diagnostic and prognostic function of lncRNA XIST in CRC chemotherapy, we then investigated the experimental role of XIST on how it can exert the pro-oncogenic role for CRC chemoresistance. CRC cell lines HT29 and HCT116 were sustained treated with 5FU with a concentration from low dose to relatively high dose ($2 \mu\text{g/mL}$) to establish the HT29 5FU-R cell line and HCT116 5FU-R cell line (Figure 4A). The established 5FU-resistant cells were maintained and exposed to $2 \mu\text{g/mL}$ 5FU unless otherwise indicated. As shown in Figure 4B, both HT29 5FU-R and HCT116 5FU-R cells showed elevated cell viability compared with HT29 and HCT116 parental cells when incubated with $2 \mu\text{g/mL}$ concentration of 5FU. On the other hand, the dose-effect curve indicated that the IC_{50} of 5FU on HT29 5FU-R cells was $11.50 \mu\text{g/mL}$, while the IC_{50} of 5FU on HT29 parental cells was $1.85 \mu\text{g/mL}$, which means that the HT29 5FU-R cells was 6.2 times the ability of 5FU resistance of HT29 parental cells. Similarly,

the HCT116 5FU-R cells was 4.7 times the ability of 5FU resistance of HCT116 parental cells ($10.95 \mu\text{g/mL}$: $2.33 \mu\text{g/mL}$, Figure 4C). After the 5FU-resistant CRC sub-lines were established, we determined the XIST expression level, and found that XIST was significantly up-regulated in HT29 5FU-R and HCT116 5FU-R cells compared with wild-type HT29 and HCT116 cells, respectively (Figure 4D).

Knockdown of XIST reverses chemoresistance in 5FU resistant cells

After having found the up-regulation of XIST in both 5FU resistant patients and cell lines, we subsequently determined the role of XIST in CRC resistance, we silenced XIST expression in CRC cell lines by small interfering RNA. As shown in Figure 5A, the knockdown effect was best using si-XIST-3 compared to si-XIST-1 and si-XIST-2. Thus we choose si-XIST-3 for further experiments. Our results showed that cell viability was significantly damaged when XIST was silenced in HT29 5FU-R and HCT116 5FU-R cells after being incubated with 5FU (Figure 5B). This suggests that XIST knockdown reverses the 5FU resistance in CRC cells.

LncRNA XIST inhibits 5-FU-induced cell death through promoting TS expression

To directly validate that XIST induces 5-FU resistance in CRC cells, we treated parental HT29 and HCT116 cells with a concentration gradient from 0-5 $\mu\text{g/mL}$ 5-FU after being transfected with p-XIST or negative control. The concentration-effect curve showed that p-XIST transfection significantly restrained the 5FU-induced cell death compared with the negative control in both HT29 and HCT116 cells (Figure 6A). Additionally,

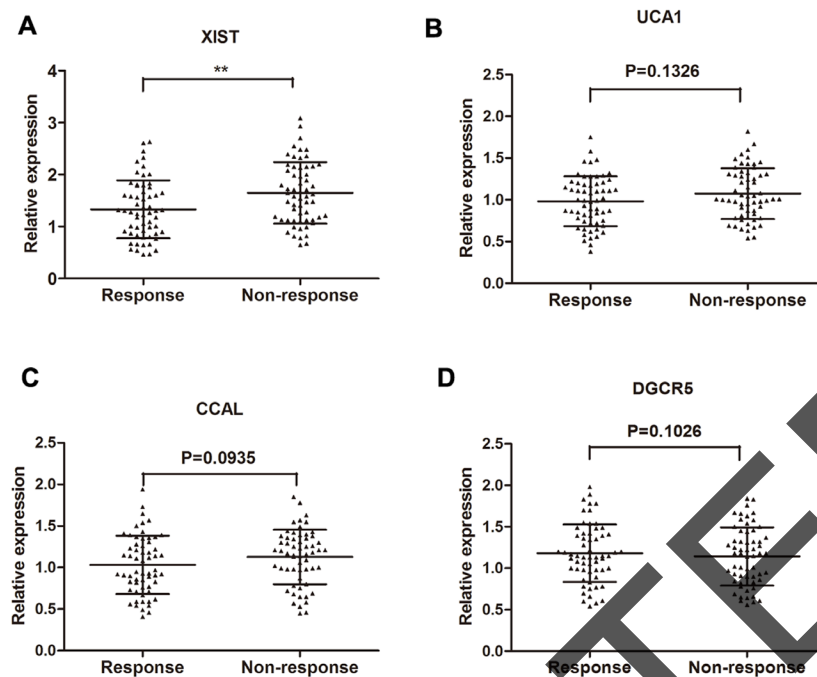


Figure 2: Concentrations of four identified serum lncRNAs in CRC patients showing response (n=60) and non-response (n=60) to 5FU treatment using RT-qPCR assay in validation cohort (A-D). Error bars represent median $\pm$ SD (Standard Deviation). **, P<0.01 by Mann-Whitney U test.

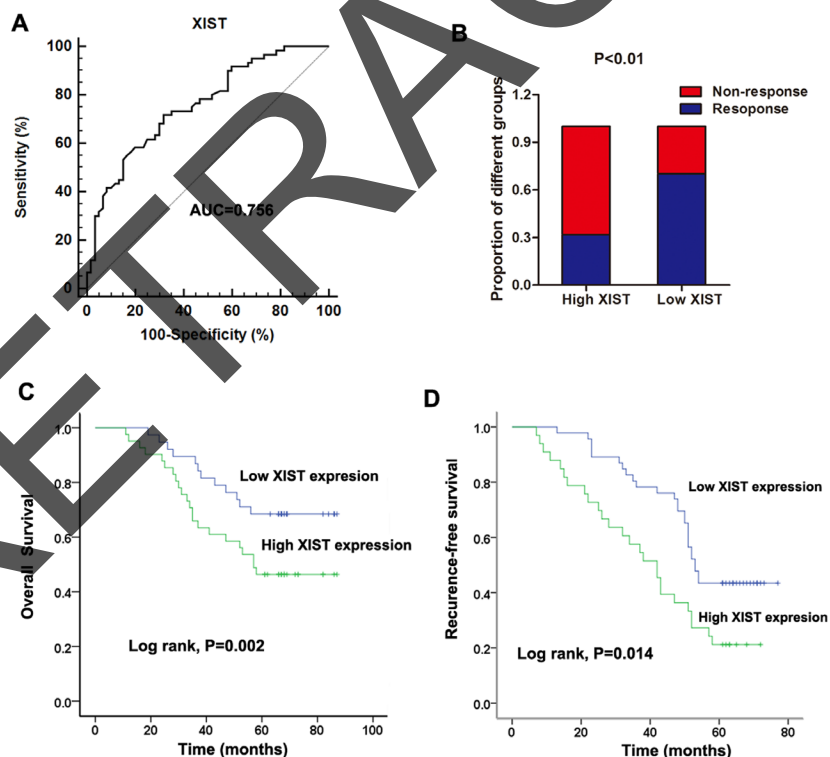


Figure 3: Increased serum XIST expression was associated with poor response to 5FU treatment in CRC patients. (A) ROC curves for differentiating the responding patients from non-responding patients of CRC using XIST expression in validation cohort. (B) The proportion of patients that responded to 5FU treatment was significantly lower in the high XIST expressing group than in the low expressing group. (C-D) Kaplan-Meier curves for OS (C) and RFS (D) according to serum levels of XIST in CRC patients in validation cohort.

Table 3: Univariate and multivariate Cox proportional hazards regression model analysis of factors for OS in patients with CRC in validation cohort

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95%CI	P value
Gender	1.023	0.609-2.027	0.694			
Age	1.508	0.732-2.893	0.192			
Tumor size	1.667	0.545-2.831	0.220			
Differentiation	1.891	1.034-3.257	0.077			
Local invasion	1.548	0.948-2.769	0.144			
Distant metastasis	2.625	1.577-3.836	0.010	2.701	1.495-4.432	0.008
TNM stage	2.257	1.144-3.506	0.037	2.209	1.112-3.417	0.043
Serum XIST level	1.927	0.381-2.234	0.005	1.990	0.378-2419	0.007

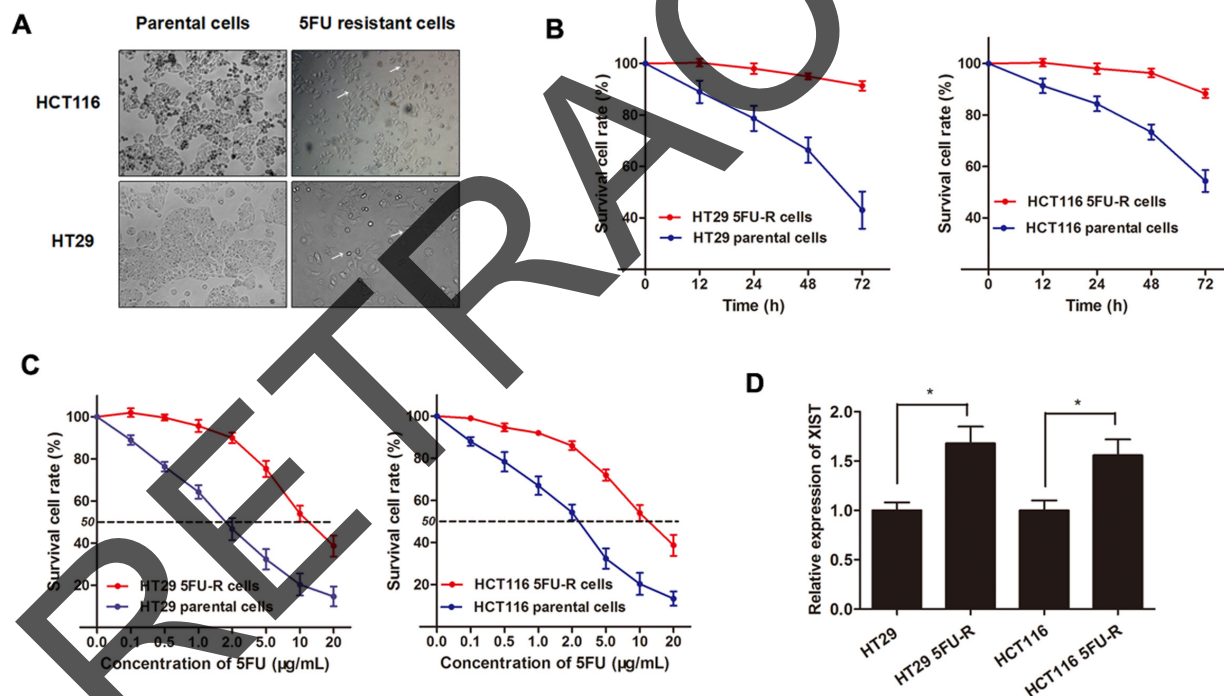


Figure 4: XIST is up-regulated in 5FU-resistant CRC cell lines. (A) The CRC cell line HT29 and HCT116 that had acquired resistance to 5FU at the clinically relevant concentration of 2 μg/mL were built as described in Methods. The built 5FU-R cells induced specific morphologic changes consistent with epithelial-mesenchymal transition (EMT), including loss of cell polarity causing a spindle-cell morphology, increased intercellular separation signifying loss of intercellular adhesion, and increased formation of pseudopodia (arrows). (B) Both HT29 5FU-R and HCT116 5FU-R cells showed elevated cell viability compared to HT29 and HCT116 parental cells when incubated with culture medium containing 2 μg/mL concentration of 5FU. (C) The concentration-effect curve indicated that the IC_{50} values of 5FU for HT29 5FU-R (11.5 μg/mL) and HCT116 5FU-R cells (10.95 μg/mL) were significant higher than that for HT29 (1.85 μg/mL) and HCT116 parental cells (2.33 μg/mL). (D) The expression of XIST in HT29 5FU-R and HCT116 5FU-R cells were significantly higher than that in HT29 and HCT116 parental cells, respectively. Error bars represent SD. *, $P < 0.05$ by Mann-Whitney U test.

the IC_{50} values for 5-FU were 1.79 and 1.93 times higher in both cells after overexpression of XIST with p-XIST than that in non-transfected cells, respectively. As 5FU suppresses tumor progression mainly by targeting TS enzyme, we suspected that XIST may induce 5-FU resistance through influencing the TS expression in CRC. As expected, western blotting showed that p-XIST significantly promoted while si-XIST suppressed the expression of TS enzyme (Figure 6B). More importantly, Immunofluorescence analysis also verified this negative

regulation mode in HT29 cells (Figure 6C). Thus, we conclude that XIST could induce 5-FU resistance in CRC cells through promoting TS enzyme expression.

DISCUSSION

CRC is one of the most malignant tumors in both males and females all around the world. Despite recent therapeutic regimens that have significantly increased

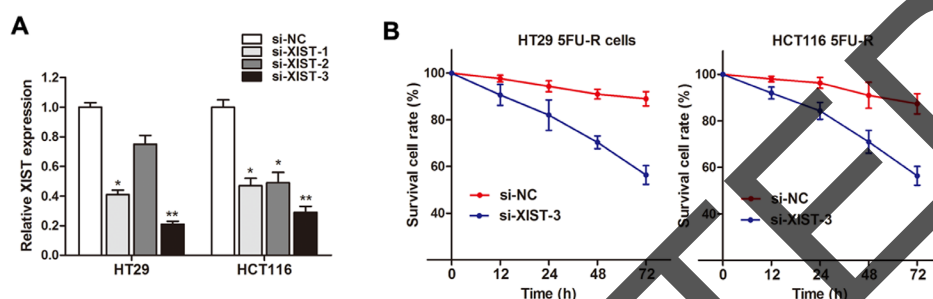


Figure 5: Knockdown of XIST reverses 5FU resistance in 5FU resistant CRC cells. (A) XIST expression level was significantly decreased after transfection of si-XIST in both HT29 5FU-R and HCT116 5FU-R cells. (B) Cell viability was significantly damaged when XIST was silenced in HT29 5FU-R and HCT116 5FU-R cells compared with negative control, respectively. Error bars represent SD. *, $P < 0.05$ and **, $P < 0.01$ by Mann-Whitney U test.

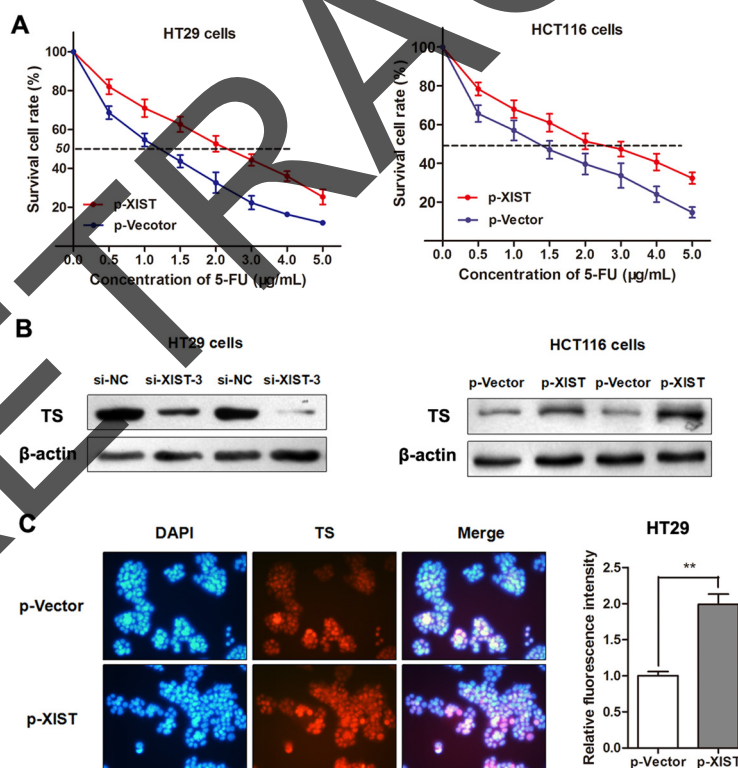


Figure 6: LncRNA XIST restrains 5-FU-induced cell cytotoxicity through promoting TS expression. (A) The dose-effect curve showed that p-XIST transfection inhibited the 5FU-induced cell death compared with the p-Vector control in HT29 and HCT116 cells. (B) Western blotting showed that TS protein was silenced by si-XIST-3 while up-regulated by p-XIST. (C) Immunofluorescence assay showed that enhanced XIST promoted the expression level of TS in HT29 cells. **, $P < 0.01$ by Mann-Whitney U test.

survival in metastatic disease, invariably, nearly all tumors become chemoresistant [21]. Thus, searching new therapeutic approach and targets, and better understanding the pathway related to chemoresistance is essential for improving the prognosis of CRC patients. In our study, high-throughput HiSeq sequencing was firstly employed to search for potential lncRNAs that may help improving the efficacy of 5FU treatment in CRC. Then, the selected lncRNAs were then detected and validated in different specimens such as primary tissues and serums from patients that have received 5FU based therapy, which can assure a relative high accuracy of the date. lncRNA XIST was then screened out as the most promising lncRNA among the candidates, and its high diagnostic value was also observed in view of high AUC, diagnostic sensitivity and specificity by ROC curves. Additionally, Kaplan-Meier analysis suggested that high XIST expression was correlate with poor chemoresponse and survival rate for CRC patients that received 5FU based therapy. More importantly, we logically demonstrate that XIST inhibits 5-FU-induced cell cytotoxicity by the promotion of TS enzyme expression in CRC cell lines.

Large-scale complementary DNA cloning projects have identified that majority of the mammalian genome is transcribed, and only minority of these transcripts represent protein-coding genes. Like other solid tumors, CRC is thought to initiate and progress through a series of genetic and epigenetic alterations [22]. The advance in sequencing technologies has facilitated the use of genome sequencing for cancer genome studies, including CRC genomes [23]. However, this sequencing technologies are not well studied in chemoresistance in cancers. In current study, we sought to identify one or more lncRNAs that may have function in the formation of resistance to the common drugs, such as 5-FU. As we know, one lncRNA may expressed different levels in different samples and different diseases. Thus, different clinical materials were used to ensure that the selected lncRNAs are qualified to the use in clinical practice. Moreover, we enrolled a relatively large sample group to ensure the steady expression of candidate lncRNAs. With this unabridged research strategy, we identified only one significantly altered lncRNA, XIST. Other three lncRNAs (UCA1, CCAL, DGCR5) were also included by the first screening but excluded by the validation analysis. Moreover, these three lncRNAs were not reported to be involved in the 5FU resistance in CRC.

Evidence has shown that lncRNAs may serve as effective therapeutic targets for cancer treatment, including breast cancer, prostate cancer, colon cancer, and HCC [24]. However, only a few lncRNAs have been functionally characterized. lncRNA XIST is required for transcriptional silencing of one X-chromosome during female mammal development. It plays a significant role in the differentiation, proliferation, and genome maintenance of human cells [25] and abnormal expression often

contributes to the development of human cancer [26]. For instance, lncRNA XIST expression is up-regulated in glioma tissues and promotes cell proliferation and invasion [27]. And its overexpression is highly associated with occurrence, growth, invasion, and metastasis in ovarian cancer [28]. Moreover, Huang et al revealed that XIST expression is associated with responses of ovarian cancer to chemotherapy [29]. However, the role of lncRNA XIST in CRC chemoresistance is still poorly understood. Our results clearly showed that the ratio of CRC patients that showed chemoresponse to 5FU treatment is dramatically higher in the low expressing XIST patient group than in the high XIST level group. Most importantly, patients with high serum XIST expression were suspected to have lower overall survival rate and recurrence-free survival rate after having received 5FU treatment. Collectively, we reveal that XIST is a lncRNA highly expressed in chemoresistant CRC patients and closely correlated to objective response to 5FU treatment.

Based on the above observations, we then sought to verify the underlying regulatory pathway that may explain those findings. By establishing two 5FU resistant cell lines, we revealed that XIST expression level was significantly increased in 5FU resistant cells compared with the parental cells. More importantly, knockdown of XIST partially reversed the 5FU resistance while enhanced XIST expression significantly suppressed 5FU-induced cell cytotoxicity in CRC cell lines, leading to chemoresistance. 5FU exerts its function of anti-tumor effect by creating fluorinated nucleotides by based on multiple mechanisms. Fluorinated nucleotides are incorporated into DNA in place of thymidine, thus preventing DNA replication and inducing cell death. Moreover, 5FU is a prodrug that experienced several intracellular enzymatic steps before it can be converted to its active metabolites, such as fluorodeoxyuridine monophosphate (FUDR-MP). FUDR-MP is the primary active metabolite that inhibits the enzyme TS, a key enzyme in the creation of the DNA nucleotide thymidine. TS functions during the reductive methylation of deoxyuridine monophosphate to deoxythymidine monophosphate, with low level of folate 5,10-methylenetetrahydrofolate as the methyl donor [30]. Thus, TS has been well accepted as one of the most important target of common chemotherapy regimens, such as 5FU and irinotecan [31]. As expected, TS enzyme was dramatically silenced by overexpression of XIST while up-regulated by XIST knockdown. These data indicate that lncRNA XIST can induce 5FU resistance in CRC cells through promoting the expression of TS enzyme.

To conclude, our integrated research strategies have demonstrated that lncRNA XIST expression is up-regulated in CRC patients who are resistant to 5FU treatment, and closely associated with chemoresponse status to 5-FU treatment. We also reveal that XIST can promote 5FU chemoresistance and influence 5FU-induced cell cytotoxicity through promoting TS expression. These

indicate that overexpression of XIST confers a potent poor therapeutic efficacy. Thus, XIST may be a potential functional biomarker and therapeutic target in CRC patients.

MATERIALS AND METHODS

Clinical samples

A multiphase, case-control study was designed to identify lncRNAs as potential biomarkers for differentiating chemo-response to 5FU therapy in CRC. Tumor response status was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 criteria and was assigned to patients with complete or partial response (CR and PR, respectively) and stable or progressive disease (SD and PD, respectively) in tumor measurements confirmed by repeat studies performed no less than four weeks after the criteria for response was first met. Briefly, 268 patients diagnosed with CRC but without other diseases were recruited from Hospital of Shupyk National Medical Academy between June 2010 and July 2013. All these participants were allocated to three phases. In the initial screening phase, tissue samples pooled from four patients showing response (CR and PR) and four patients showing no response (SD and PD) were subjected to Hiseq sequencing, to identify lncRNAs significantly differentially expressed. In the training phase, the candidate lncRNAs were tested with RT-qPCR in an independent cohort of primary tissues from 70 CRC patients responding to 5FU treatment and 70 patients showing no response to treatment. In the validation phase, another independent group of 120 CRC patients who provided serum samples were enrolled. Among these patients, there are 60 patients showing response to 5FU treatment while the other 60 patients showing no response.

All the patients were pathologically confirmed and the clinical tissue samples were collected before chemotherapy was started. They were classified according to the WHO criteria and staged according to the tumor-node-metastasis (TNM) classification. Fresh tumor tissues were immediately cut from the resected CRC tissue and kept at -80°C until RNA extraction. Venous blood was collected and centrifuged at 4000 rpm for 10 min, within 2 h. The supernatant fluids were then collected and further centrifuged at 12,000 rpm for 15 min to completely remove the cell debris. The whole process was strictly controlled to avoid hemolysis, and the supernatant serum was stored at -80°C, until analysis. Overall survival (OS) was updated on 1 February 2012 and was defined as the time from inclusion to death for any reason. Recurrence free survival (RFS) was defined as the time from inclusion to recurrence or metastasis progression. Written informed consent was obtained from all patients according to local ethical regulations of the Ethics Committee of Shupyk National Medical Academy.

Hiseq sequencing

The Hiseq sequencing work was performed by Kangchen Bio-tech (Shanghai, China). Total tissue RNA was extracted by one-step extraction using a Trizol kit (Life Technologies, USA), and the purity and quantity of RNA were determined by UV spectrophotometry. cDNA library construction and sequencing were performed according to previously described methods [32]. Briefly, after extraction of total RNA, ribosomal RNA was separated to isolate as ncRNA as possible. RNA containing poly(A) was then removed. RNA fragments were broken into short fragments randomly. The first chain of cDNA was generated using RNA fragments as templates and 6-bp random primers. Second chain of the cDNA was synthesized according to the kit's instruction (TakaRa Bio Company, Dalian, China). After purification, end repair, base A and sequencing joint adding, the generated cDNA was fragmented using uracil-N-glycosylase (UNG). cDNA fragments were chosen according to size, then PCR amplification was performed to establish the complete sequencing cDNA library. lncRNAs were sequenced using the high-throughput, high-sensitivity Hiseq 2500 sequencing platform (Illumina Company, USA). Sequencing results were analyzed and treated using Trim Galore software to dynamically remove joint sequence fragments and low-quality segments from the 3' end. FastQC software was used for quality control of the pretreated data.

Cell culture

The human CRC cell lines HT29, HCT116, and FHC (human fetal normal colonic cell) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) in 2015. CRC cell lines were maintained in RPMI 1640 (Thermo Fisher Scientific, Wilmington, DE, USA) containing 10% FBS (fetal bovine serum) (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 g/ml streptomycin (Life Technologies, Grand Island, NY, USA) at 37°C in 5% CO₂ and 95% air. Normal colon FHC cells were grown in DMEM/F12 medium with 10% FBS, 10 ng/mL cholera toxin, 5 µg/mL transferrin, 5 µg/mL insulin, 100 ng/mL hydrocortisone and additional 10 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at 37°C in 5% CO₂ and 95% air. The cell lines passed the DNA profiling test (STR).

Development of 5FU-Resistant (5FU-R) CRC cell lines

HT29 5FU-R cell line was developed by exposing parental HT29 and HCT116 cells to an initial dose of 0.1 µg/mL 5FU (Sigma-Aldrich) in RPMI 1640 plus 10% FBS. The surviving population of cells was grown to 80% confluence for three passages over 6 weeks. The cells that

survived initial 5FU treatment were then exposed to 0.5 µg/mL 5FU for three passages (8 wk) and then 1.0 µg/mL for three passages (8 wk). Finally, the 5FU concentration was increased to the clinically relevant plasma concentration of 2 µg/mL and the cells were continuously cultured in 2 µg/mL 5FU, unless otherwise indicated. For all experiments, HT29 5FU-R cells was used at no higher than 15 passages from creation.

RNA extraction and RT-qPCR

Total RNA was isolated from primary tumors or CRC cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For lncRNA and mRNA detection, RNA was reverse-transcribed with a random primer using a TaKaRa Reverse Transcription Kit and amplified with SYBR Green Kit (Takara Bio Company) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) with the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as internal controls. The $2^{-\Delta\Delta C_t}$ method was used to determine the relative quantification of gene expression levels. The primer sequences are as follows: XIST (Forward): 5'-CTCTCCATTGGGTTTCAC-3', (Reverse): 5'-GCGGCAGGTCTTAAGAGATGAG-3'; GAPDH (Forward): 5'-GCACCGTCAAGGCTGAGAAC-3', (Reverse): ATGGTGGTGAAGACGCCAGT.

Cell transfection

The small interfering RNA (siRNA) that specifically target human lncRNA XIST were designated as si-XIST (Genechem corporation, Shanghai, China). The XIST overexpression plasmid (p-XIST) or control vector (p-Vector) was purchased from Genechem. Forty-eight hours after planting CRC cells into 24-well plate, 100 nM of si-XIST or p-XIST as well as negative controls were transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The oligoribonucleotide sequences are si-XIST-1 (sense): 5'-GCAA AUGAAAGCUACCAAU3', (antisense): 5'-AUUGGUAGCUUUCAUUUGC3'; si-XIST-2 (sense): 5'-GCACAAUAUCUUUGAACUA3', (antisense): 5'-UAGUUCAAAGAUUUGUGC3'; si-XIST-3 (sense): 5'-CUAGAAUCCUAAAGGCAAA3', (antisense): 5'-UUUGCCUUUAGGAUUCUAG3'.

Cell viability assay

Cell viability was quantified by using the Cell Counting Kit-8 (CKK-8, Beyotime Corporation, Shanghai, China). Briefly, 100 µl of cells from the different transfection groups were seeded onto a 96-well plate at a concentration of 2000 cells per well and were incubated at 37°C. At different time point, the optical density was measured at 450 nm using a microtiter plate reader, and the rate of cell survival was expressed as the absorbance.

The results represent the mean of three replicates under the same conditions. Additionally, the concentration-dependent curves were generated based on the cell viability after the cells were cultured for 72h at different concentration of 5-FU. The results represent the mean of three replicates under the same conditions.

Immunofluorescence analysis

HT29 cells were grown to 40% to 50% confluence and then transfected with 100 nM of p-XIST or p-Vector. After 48 hours of incubation, the cells were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 (Sigma-Aldrich) for 20 minutes. The cells were then blocked with 10% goat serum in PBS for 1 h. Cells were incubated with primary anti-TS (1:100, Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C and then incubated with the appropriate rhodamine-conjugated secondary antibody for 1 h. The cells were then washed and incubated with DAPI (4',6-diamidino-2-phenylindole, Invitrogen) for nuclear staining. The slides were visualized for immunofluorescence with a laser scanning Olympus microscope.

Western blot and antibodies

The primary antibodies used for western blot were rabbit anti-human TS antibody (1:1000; Cell Signaling Technology) and rabbit anti-human β-actin antibody (1:1000; Cell Signaling Technology). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the secondary antibodies. Cell lysates in 1× SDS loading buffer (60 mM Tris-HCl, pH 6.8; 2% SDS; 20% glycerol; 0.25% bromophenol blue; and 1.25% 2-mercaptoethanol) were incubated at 100°C for 10 min to facilitate sample loading for conventional western blotting analysis. The relative protein levels were quantified using densitometry with a Gel-Pro Analyzer (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Kolmogorov-Smirnov test was used to determine the normality of the distribution of data in each group. Data were presented as median (interquartile range). Differences in cell growth curves and cell cytotoxicity curves were determined by repeated measures analysis of variance. The differences of lncRNA or mRNA expression level between different groups were analyzed by the Mann-Whitney U-test or Kruskal-Wallis test. A log-rank test was used to analyze the statistical differences in survival as deduced from Kaplan-Meier curves. Receiver operating characteristic (ROC) curves were established to discriminate CRC responding patients from non-responding patients. Count dates were described as frequency and examined using Fisher's exact test. All

differences were regarded as statistically significant when $P < 0.05$. Statistical analyses were performed with GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA).

CONFLICTS OF INTEREST

The authors report that there are no conflicts of interest.

REFERENCES

- Li PL, Zhang X, Wang LL, Du LT, Yang YM, Li J, Wang CX. MicroRNA-218 is a prognostic indicator in colorectal cancer and enhances 5-fluorouracil-induced apoptosis by targeting BIRC5. *Carcinogenesis*. 2015;36:1484-1493.
- Han D, Wang M, Ma N, Xu Y, Jiang Y, Gao X. Long noncoding RNAs: novel players in colorectal cancer. *Cancer Lett*. 2015;361:13-21.
- Tomida C, Aibara K, Yamagishi N, Yano C, Nagano H, Abe T, Ohno A, Hirasaka K, Nikawa T, Teshima-Kondo S. The malignant progression effects of regorafenib in human colon cancer cells. *J Med Invest*. 2015;62:195-198.
- Alberts SR, Horvath WL, Sternfeld WC, Goldberg RM, Mahoney MR, Dakhil SR, Levitt R, Rowland K, Nair S, Sargent DJ, Donohue JH. Oxaliplatin, fluorouracil, and leucovorin for patients with unresectable liver-only metastases from colorectal cancer: a North Central Cancer Treatment Group phase II study. *J Clin Oncol*. 2005;23:9243-9249.
- Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pitor HC, Alberts SR. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. *J Clin Oncol*. 2004;22:23-30.
- Hector S, Bolanowska-Higdon W, Zdanowicz J, Hitt S, Pendyala L. *In vitro* studies on the mechanisms of oxaliplatin resistance. *Cancer Chemother Pharmacol*. 2001;48:398-406.
- Ahmad S. Platinum-DNA interactions and subsequent cellular processes controlling sensitivity to anticancer platinum complexes. *Chem Biodivers*. 2010;7:543-566.
- Landriscina M, Maddalena F, Laudiero G, Esposito F. Adaptation to oxidative stress, chemoresistance, and cell survival. *Antioxid Redox Signal*. 2009;11:2701-2716.
- Sau A, Pellizzari Tregno F, Valentino F, Federici G, Caccuri AM. Glutathione transferases and development of new principles to overcome drug resistance. *Arch Biochem Biophys*. 2010;500:116-122.
- Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer*. 2002;2:48-58.
- Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski F, Aken BL, Barrell D, Zadissa A, Searle S, Barnes I, Bignell A, Boychenko V, et al. GENCODE: the reference human genome annotation for The ENCODE Project. *Genome Res*. 2012;22:1760-1774.
- Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, Stadler PF, Hertel J, Hackermuller J, Hofacker IL, Bell I, Cheung E, Drenkow J, et al. RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science*. 2007;316:1484-1488.
- Lai Y, Xu P, Li Q, Ren D, Wang J, Xu K, Gao W. Downregulation of long noncoding RNA ZMAT1 transcript variant 2 predicts a poor prognosis in patients with gastric cancer. *Int J Clin Exp Pathol*. 2015;8:5556-5562.
- Zhao B, Hou X, Zhan H. Long non-coding RNA PCAT-1 over-expression promotes proliferation and metastasis in non-small cell lung cancer cells. *Int J Clin Exp Med*. 2015;8:18482-18487.
- Yin DD, Liu ZJ, Zhang E, Kong R, Zhang ZH, Guo RH. Decreased expression of long noncoding RNA MEG3 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Tumour Biol*. 2015;36:4851-4859.
- Yang F, Liu YH, Dong SY, Ma RM, Bhandari A, Zhang XH, Wang OC. A novel long non-coding RNA FGF14-AS2 is correlated with progression and prognosis in breast cancer. *Biochem Biophys Res Commun*. 2016;470:479-483.
- Ma Y, Yang Y, Wang F, Moyer MP, Wei Q, Zhang P, Yang Z, Liu W, Zhang H, Chen N, Wang H, Wang H, Qin H. Long non-coding RNA CCAL regulates colorectal cancer progression by activating Wnt/beta-catenin signalling pathway via suppression of activator protein 2 α . *Gut*. 2016;65:1494-1504.
- Ji Q, Liu X, Fu X, Zhang L, Sui H, Zhou L, Sun J, Cai J, Qin J, Ren J, Li Q. Resveratrol inhibits invasion and metastasis of colorectal cancer cells via MALAT1 mediated Wnt/beta-catenin signal pathway. *PLoS One*. 2013;8:e78700.
- Chen T, Yang P, Wang H, He ZY. Silence of long noncoding RNA PANDAR switches low-dose curcumin-induced senescence to apoptosis in colorectal cancer cells. *Oncotargets Ther*. 2017;10:483-491.
- Bian Z, Jin L, Zhang J, Yin Y, Quan C, Hu Y, Feng Y, Liu H, Fei B, Mao Y, Zhou L, Qi X, Huang S, et al. LncRNA-UCA1 enhances cell proliferation and 5-fluorouracil resistance in colorectal cancer by inhibiting miR-204-5p. *Sci Rep*. 2016;6:23892.
- Li P, Zhang X, Wang H, Wang L, Liu T, Du L, Yang Y, Wang C. MALAT1 is associated with poor response to oxaliplatin-based chemotherapy in colorectal cancer patients and promotes chemoresistance through EZH2. *Mol Cancer Ther*. 2017;16:739-751.
- Fearon ER. Molecular genetics of colorectal cancer. *Annu Rev Pathol*. 2011;6:479-507.

23. Kim TM, Lee SH, Chung YJ. Clinical applications of next-generation sequencing in colorectal cancers. *World J Gastroenterol*. 2013;19:6784-6793.
24. Carpenter S. Long noncoding RNA: novel links between gene expression and innate immunity. *Virus Res*. 2016;212:137-145.
25. Ma MZ, Chu BF, Zhang Y, Weng MZ, Qin YY, Gong W, Quan ZW. Long non-coding RNA CCAT1 promotes gallbladder cancer development via negative modulation of miRNA-218-5p. *Cell Death Dis*. 2015;6:e1583.
26. McHugh CA, Chen CK, Chow A, Surka CF, Tran C, McDonel P, Pandya-Jones A, Blanco M, Burghard C, Moradian A, Sweredoski MJ, Shishkin AA, Su J, et al. The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. *Nature*. 2015;521:232-236.
27. Yao Y, Ma J, Xue Y, Wang P, Li Z, Liu J, Chen L, Xi Z, Teng H, Wang Z, Li Z, Liu Y. Knockdown of long non-coding RNA XIST exerts tumor-suppressive functions in human glioblastoma stem cells by up-regulating miR-152. *Cancer Lett*. 2015;359:75-86.
28. Ren C, Li X, Wang T, Wang G, Zhao C, Liang T, Zhu Y, Li M, Yang C, Zhao Y, Zhang GM. Functions and mechanisms of long noncoding RNAs in ovarian cancer. *Int J Gynecol Cancer*. 2015;25:566-569.
29. Huang KC, Rao PH, Lau CC, Heard E, Ng SK, Brown C, Mok SC, Berkowitz RS, Ng SW. Relationship of XIST expression and responses of ovarian cancer to chemotherapy. *Mol Cancer Ther*. 2002;1:769-776.
30. Danenberg PV. Thymidylate synthetase - a target enzyme in cancer chemotherapy. *Biochim Biophys Acta*. 1977;473:73-92.
31. Chu E, Callender MA, Farrell MP, Schmitz JC. Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. *Cancer Chemother Pharmacol*. 2003;52:S80-S89.
32. Iyer MK, Niknafs YS, Malik R, Singhal U, Sahu A, Hosono Y, Barrette TR, Prensner JR, Evans JR, Zhao S, Poliakov A, Cao X, Dhanasekaran SM, et al. The landscape of long noncoding RNAs in the human transcriptome. *Nat Genet*. 2015;47:199-208.

RETRACTED