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Potential role of TET2 in gastric cancer cisplatin resistance

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ABSTRACT

Gastric cancer (GC) represents one of the most predominant malignancies with high incidence and mortality rates. Although traditional chemotherapeutics, including cisplatin are effective in the treatment of GC, patients often develop drug resistance in clinic. The present study aimed to explore the underlying mechanism of cisplatin-induced drug resistance in GC. The potential role of DNA demethylase ten-eleven translocation-2 (TET2) in modulating cisplatin resistance of GC cells was investigated. It was observed that TET2 was significantly decreased in cisplatin resistance SGC7901/DDP cells compared with non-resistant cells and TET2 overexpression markedly reduced the tolerance to cisplatin. Additionally, evidence was provided that TET2 regulated interleukin-6 levels in the tumor microenvironment through histone acetylation and therefore served an important role in the development of cisplatin resistance in GC cells. Taken together, the results suggested that TET2-mediated cisplatin resistance may represent a novel mechanism of drug resistance in GC cells and may offer novel treatment approaches.

1. Introduction

Cancer is a disease with epigenetic alterations and disordered gene expression [9]. Currently, cancer treatment includes radio- and chemotherapy, surgery and targeted therapies [14,19]. However, the prognosis for most patients with cancer is not satisfactory. Among the various human tumors, gastric cancer (GC) represents a predominant malignancy. The high frequency of incidence and mortality of GC has become a public health threat and socioeconomic burden [12,16].

DNA methylation is an important epigenetic modification, which has significant effect on embryogenesis, tumorigenesis and various other pathophysiological processes [4]. Aberrant DNA methylation patterns are a characteristic feature of diverse malignancies [7]. The ten-eleven translocation (TET) proteins are demethylases, which specifically modify DNA by converting 5-methylcytosine to 5-hydroxymethylcytosine [10]. In addition to their role in modulating DNA methylation, accumulating evidence has demonstrated that TET proteins are novel tumor suppressors in various cancers [5,9,15]. It has been reported that TET2 represses the transcription of specific genes *via* histone deacetylation and the loss of TET2 results in the upregulation of several inflammatory mediators, conferring the ability of TET2 to regulate inflammation and immunity [21].

Drug resistance is a common cause of tumor treatment failure and it is generally accepted that the tumor microenvironment is closely associated with drug resistance [2,18]. Tumors influence the microenvironment by releasing extracellular cytokines, promoting tumor growth and inducing drug resistance [11]. However, to the best of our knowledge, the roles of TET proteins, in particular TET2, in drug resistance remains unknown.

Here, based on the documented involvement of TET2 in inflammation and cancer, evidence that TET2 regulated interleukin (IL)-6 levels in the tumor microenvironment through histone acetylation was provided and it was suggested that TET2 may serve an important role in the development of drug resistance of GC cells.

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2. Materials and methods

2.1. Cell culture

The human GC cell line SGC7901 was purchased from the Chinese Academy of Medical Sciences Cell Bank (Beijing, China). All cells were cultured in 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. To establish cisplatin-resistant SGC7901 cells (SGC7901/DDP), SGC7901 cells were first treated with 0.01 μg/ml cisplatin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and then with increasing concentrations of cisplatin until the drug resistance index (RI) was > 5. To maintain the cisplatin-resistant phenotype, cisplatin (final concentration, 1 μg/ml) was added to the culture medium of SGC7901/DDP cells.

2.2. Cell transfection

TET2 overexpression plasmid (pcDNA3.1) was synthesized and purchased from Genechem (Shanghai Genechem, China). All cell transfections assays were performed by using lipofectamine 2000 according to the manufacturer's instructions (ThermoFisher Scientific, 11668027, USA).

2.2.1. Tissue specimens and blood samples

A total of 64 GC and paired non-carcinoma tissues, along with serum samples were collected between February 2015 and September 2015 at the General Hospital of Heilongjiang Province Land Reclamation Bureau (Harbin, China). The study included 35 male patients and 29 female patients, with a mean age of 58 years. All cases were diagnosed by pathology and immunohistochemistry. Besides, the blood samples were obtained from GC patients before cisplatin resistance and after cisplatin resistance. The basis for defining cisplatin resistance in GC patients is that the patient developed disease progression or recurrence during chemotherapy or within 3 months after the end of chemotherapy. The present study was approved by the Ethics Committee of the General Hospital of Heilongjiang Province Land Reclamation Bureau (Harbin, China) and written informed consent was obtained from all patients.

2.3. Proliferation assay

Cell Counting kit-8 (CCK-8) assays were used to determine proliferation according to the manufacturer's instructions (Solarbio, CA1210, Beijing, China). Briefly, cells were seeded in triplicate in 96-well plates at 1×10^4 cells/well and maintained for 24 h. Following treatment, 10 μl CCK-8 working solution was added to each well and plates were incubated in 37 °C for 2 h. Absorbance was recorded at 450 nm using an Easy Reader 340 AT (SLT-Lab Instruments, Inc., Ronkonkoma, NY, USA). The experiment was independently repeated three times.

2.4. ELISA

Peripheral blood was collected prior to and following the development of drug resistance. For the detection of IL-6 secreted by GC cells, cell supernatants were collected. IL-6 levels in serum and cell supernatants were measured using ELISA according to the manufacturer's instructions (human IL-6 ELISA kit; BMS213-2, Thermo Fisher Scientific, Inc.). Briefly, a series of IL-6 concentrations (0–2,000 pg/ml) was prepared as protein standards. The absorption in each well was determined using a microplate reader at 490 nm. Sample concentrations were calculated using the standard curve.

2.5. Immunohistochemistry

Paraffin-embedded slides (0.4 μm) were incubated with TET2 antibody (dilution, 1:200; cat no., ab230358; Abcam, Cambridge, UK) overnight at 4 °C. TET2 expression was scored based on staining intensity (0, 1+, 2+, 3+) and the percentage of TET2-positive area [0 (< 5%), 1 (5–25%), 2 (26–50%) and 3 (51–75%), 4 (> 75%). The final staining score was calculated by multiplying the individual scores (range, 0–12). Staining scores were independently evaluated by two pathologists. For further assessment, negative and weak TET2 staining cases (score index, ≤7) were defined as negative expression and TET2-positive cases (score index, > 7) as high expression.

2.6. Protein extraction and western blot assays

GC cells were homogenized with lysis buffer (Beyotime Institute of Biotechnology, P0013, Shanghai, China) and protein concentrations were detected using a NanoDrop spectrophotometer. Proteins (50 μg) were separated on 8% SDS-PAGE gels and subsequently transferred to polyvinylidene fluoride membranes (Millipore; ISEQ00010, Merck KGaA, Darmstadt, Germany), followed by blocking with 5% nonfat dried milk in PBST for 60 min at room temperature. Membranes were then incubated at 4 °C overnight with primary antibodies against TET2 (dilution, 1:1,000; cat no., ab230358; Abcam), HADC2 (dilution, 1:1,000; cat no., ab32117; Abcam), IKB-ζ (dilution, 1:1,000; cat no., ab155142; Abcam) and β-actin (dilution, 1:1,000; cat no., #3700; Cell Signaling Technology, Inc., Danvers, MA, USA). Following, the membranes were washed with PBST and incubated with Alexa Fluor labeled secondary antibodies (dilution, 1:5,000; cat nos., #A32730 and #A32732; Alexa Fluor; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Protein bands were quantified using Odyssey v1.2 (LI−COR Biosciences, Lincoln, NE, USA) by measuring the band intensity and normalizing to β-actin as an internal control.

2.7. Immunoprecipitation

For immunoprecipitation, proteins extracted from SGC7901/DDP cells and their parental cells were incubated with 30 μl protein G agarose (Pharmacia; 17-0618-01, GE Healthcare Life Sciences, Uppsala, Sweden) bound with Tet2 antibody (dilution, 1:200; cat no., ab230358; Abcam) overnight at 4 °C with gentle shaking. Bound proteins were eluted by heating to 37 °C for 10 min in SDS-PAGE sample buffer (Beyotime Institute of Biotechnology, P0013D, Shanghai, China) and centrifuged at 1,500*g* at 4 °C for 5 min. Supernatants were collected for western blot analysis.

2.8. RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Gene expression was determined by RT-qPCR. Total RNA samples were extracted from GC cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and RNA concentrations were detected by NanoDrop. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; 4368814, Thermo Fisher Scientific, Inc.). The temperature protocol for reverse transcription was as follows: 25 °C 10 min, 37 °C 120 min, 85 °C 5 min. The SYBR Green PCR Master Mix kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for qPCR assays. The temperature protocol for qPCR was as follows: 95 °C 1 min, and 40 cycles of 94 °C for 30 s, 57 °C for 35 s and 72 °C for 40 s and 72 °C for 10 m in. The primer sequences used were as follows: Tet2, forward, 5′−CCTCCTTACTCATGGTCGGATC-3′, reverse, 5′- CTGTTTCCTTCACATG CCCC-3′; GAPDH, forward, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, reverse, 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. qPCRs were performed using the ABI 7500 Fast system with ABI 7500 software v2.0.1 (Thermo Fisher Scientific, Inc.). GAPDH was used as internal control for

the gene mRNA expression levels, and the results were calculated by using the $2^{-\Delta\Delta CT}$ method.

2.9. Flow cytometry

Flow cytometry was performed to determine the apoptosis levels of SGC7901 cells according to the FITC Annexin V Apoptosis Detection Kit I (BD Bioscience, 556547, USA). Briefly, cells were centrifuged at 1,000 *g* for 5 min at room temperature following trypsinization. Then the cells were resuspended in the binding buffer provided by the FITC Annexin V Apoptosis Detection Kit I. Subsequently, 10 µl Annexin V/FITC and 5 µl propidium iodide (PI) were added to the cell suspension in the dark environment for 15 min. Finally, the samples were detected by using a flow cytometer (BD Bioscience, FACSCalibur Flow Cytometer, USA) and analyzed by FlowJo software (version 7.6.1, FlowJo LLC, USA).

2.10. Statistical analysis

Data are presented as the mean ± standard deviation and were analyzed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). Survival analysis was performed using Kaplan-Meier method and the correlation analysis between levels of IL-6 and IHC staining scores of TET2 were evaluated using Pearson's correlation coefficient. Differences between two groups were analyzed by Student's *t*-test and multiple groups were analyzed by one-way analysis of variance followed by Bonferroni's multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. TET2 is negatively correlated with IL-6 in GC

To determine the clinical relevance of IL-6 and TET2 in GC, ELISA and immunohistochemistry assays were performed. As presented in Fig. 1A, IL-6 levels in the serum were significantly elevated in patients with gastric cancer after cisplatin resistance (ADR), compared with the IL-6 levels before cisplatin resistance (BDR). Immunohistochemical staining of the paraffin-embedded human GC tissues suggested differential expression of TET2 in GC compared with normal tissues (Fig. 1D). To assess the prognostic value of TET2 expression in GC, the correlation between TET2 expression and GC prognosis was assessed using the Kaplan-Meier survival analysis. As demonstrated in Fig. 1B, the overall survival time in the TET2-low group was significantly decreased compared with the TET2-high group ($p < 0.01$). Furthermore, Pearson correlation analysis demonstrated that the expression of TET2 was negatively correlated with serum IL-6 levels (Fig. 1C). These results prompted us to conduct further research into the underlying molecular and signaling mechanisms associated with cisplatin resistance in GC.

3.2. Establishment of cisplatin-resistant cells

To establish SGC7901/DDP cells, SGC7901 cells were first treated with 0.01 µg/ml of cisplatin and cisplatin concentrations were increased in a stepwise manner until RI (resistance index) > 5. To maintain the cisplatin-resistant phenotype, cisplatin (final concentration, 1 µg/ml) was added to the culture medium. The half maximal inhibitory concentration and the RI of the SGC7901/DDP cells and their parental cells were determined by MTT assay. As illustrated in Fig. 2A, the RI of SGC7901/DDP cells was 6.19, indicating that SGC7901/DDP cells exhibited a significant resistant to cisplatin. These results were further supported by the cell growth curve presented in Fig. 2B.

3.3. Loss of TET2 retains high IL-6 expression through histone deacetylation

In addition to previously reported regulatory roles of TET2 in DNA methylation, TET2 regulates the transcription of inflammatory genes, including IL-6 through HDAC2-involved histone acetylation [13,22]. First, TET2 expression prior to and following cisplatin resistance were determined. As presented in Fig. 3A and B, mRNA and protein expression levels of TET2 were significantly reduced in SGC7901/DDP cells compared with the parental SGC7901 cells. Furthermore, the result of immunoprecipitation suggested that the amount of HDAC2 that interacted with TET2 was significantly reduced in SGC7901/DDP compared with SGC7901 cells, accompanied with increased expression of IKB-ζ (Fig. 3C), an IL-6-specific transcription factor, indicating

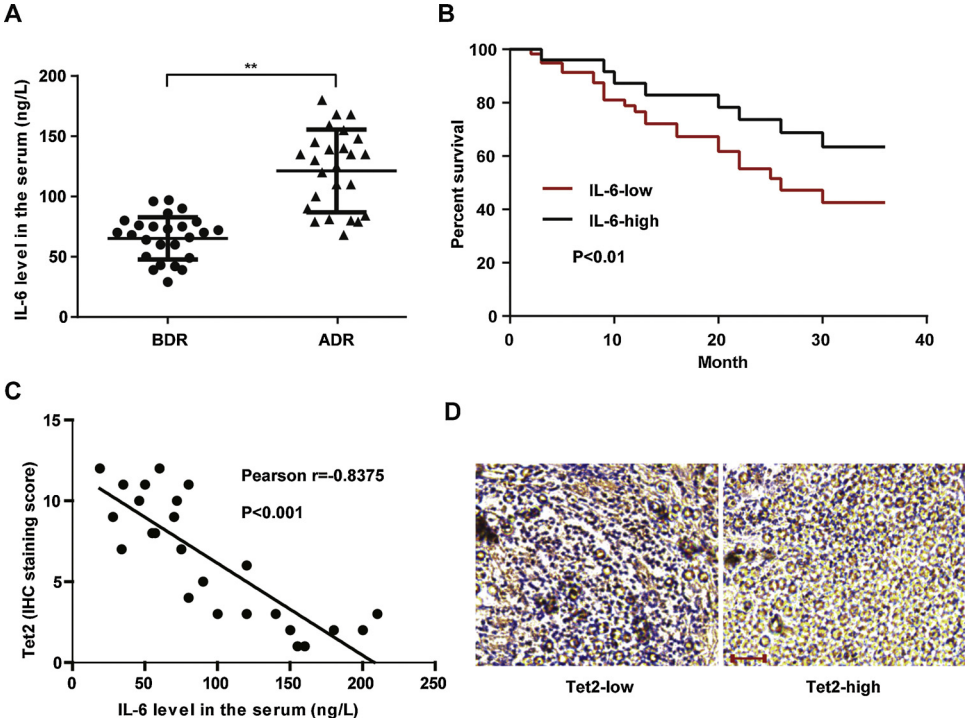

Fig. 1. The expression of TET2 and IL-6 in GC and their prognostic significance. (A) The IL-6 levels in the serum before cisplatin resistance and after cisplatin resistance in GC patients. BDR: before drug resistance, ADR: after drug resistance. **p < 0.01 *vs.* BDR. n = 25. (B) The overall survival time of the 64 patients with GC. **p < 0.01 *vs.* TET2-low. n = 64. (C) The expression of TET2 is negatively correlated with serum IL-6, determined by Pearson correlation analysis. Pearson r = -0.8375, p < 0.001, n = 25. (D) The representative image of immunohistochemistry staining TET2. Red scale bar represents 20 µm.

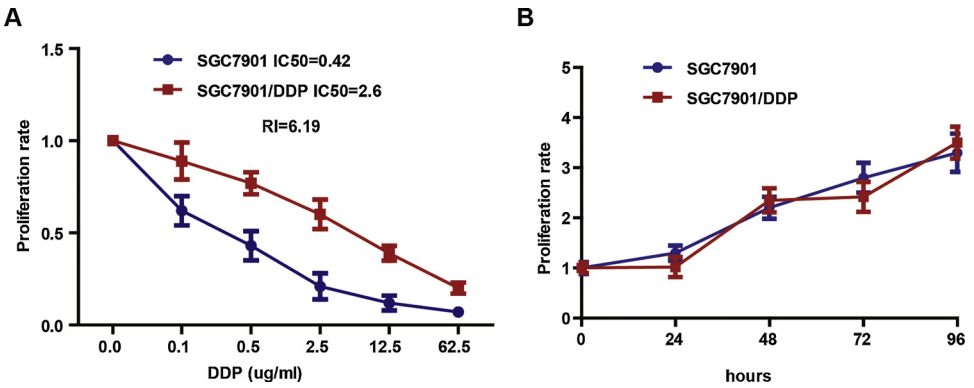

Fig. 2. The proliferation curve of SGC7901 and cisplatin resistance SGC7901/DDP. (A) The half maximal inhibitory concentration (IC50) and drug resistance index (RI) of the SGC7901/DDP cells as well as their parental SGC7901 cells determined by MTT assay. (B) The proliferation curve of SGC7901 and SGC7901/DDP determined by MTT.

potentially enhanced IL-6 expression. Interestingly, it was observed that the IL-6 level in the cell culture medium was significantly elevated in SGC7901/DDP compared with the parental SGC7901 cells (Fig. 3E).

3.4. Enhanced TET2 levels inhibit IL-6 expression and attenuate cisplatin resistance

To confirm that TET2 serves a crucial role in drug resistance, gain-of-function assays were performed following the synthesis of a TET2 overexpression plasmid and transfection of SGC7901/DDP cells. As presented in Fig. 4A and B, transfection of the TET2 overexpression

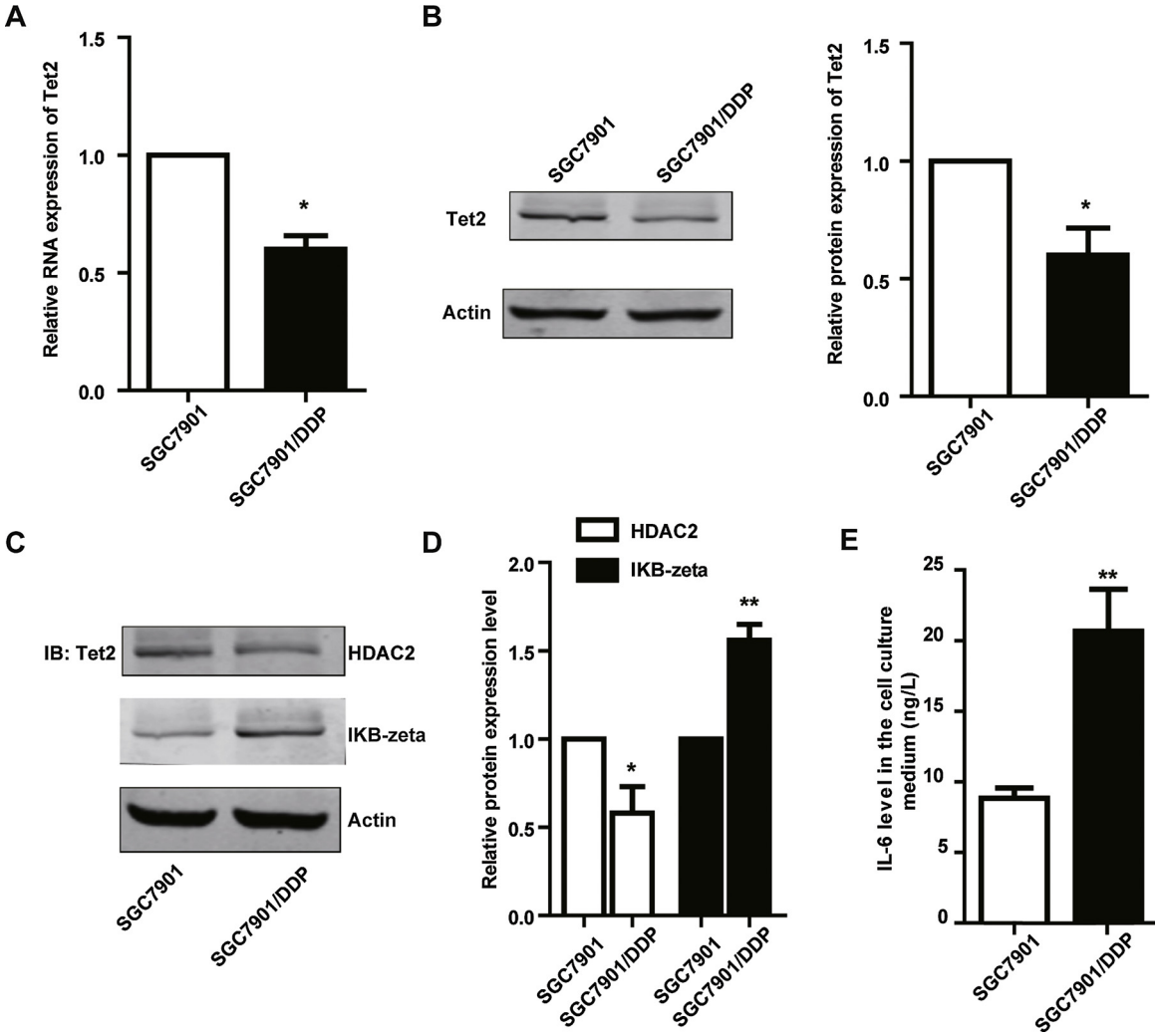

Fig. 3. The expression of TET2, IL-6 in SGC7901/DDP cells and the potential mechanism. (A) The mRNA expression of TET2 in SGC7901/DDP and their parental SGC7901 cells. *p < 0.05 *vs.* SGC7901. n = 6. (B) The protein expression of TET2 in SGC7901/DDP and their parental SGC7901 cells. *p < 0.05 *vs.* SGC7901. n = 6. (C) The immunoprecipitation result represents the reduced interaction of HDAC2 with TET2 (upper lane). And the western blot result represents the expression levels of IKB-ζ (middle lane). (D) The statistical chart represents the relative protein expression levels. *p < 0.05 or **p < 0.01 *vs.* SGC7901. n = 6. (E) The IL-6 levels in the culture medium of SGC7901 and SGC7901/DDP cells. **p < 0.01 *vs.* SGC7901. n = 6.

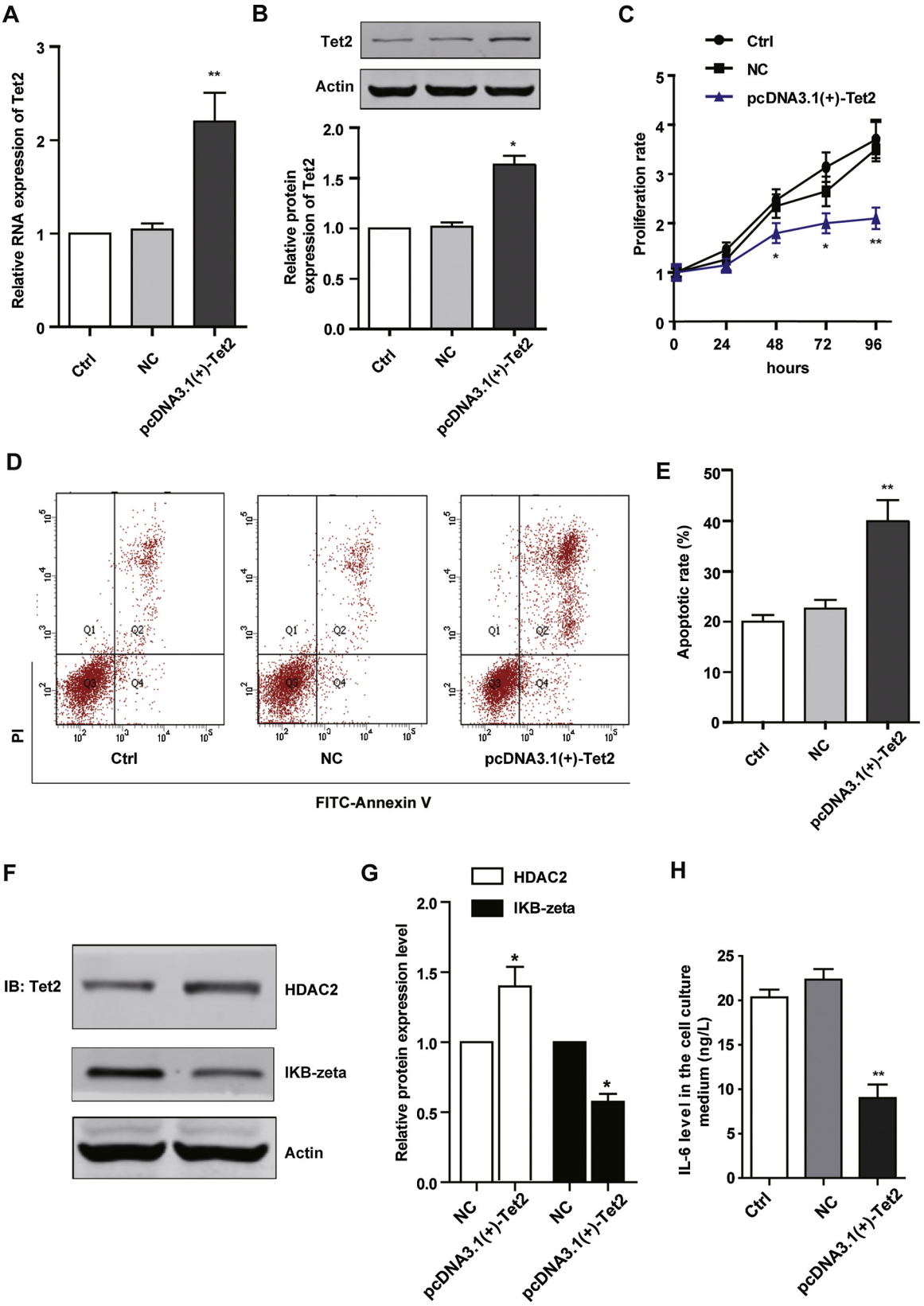

Fig. 4. Enhanced TET2 inhibits IL-6 expression and attenuates cisplatin resistance. (A) The mRNA expression of TET2 in SGC7901/DDP after transfection of TET2 overexpression plasmid. **p < 0.01 *vs*. NC. n = 6. NC: negative control. (B) The protein expression of TET2 in SGC7901/DDP in different groups and the statistical chart represents the relative protein expression levels. *p < 0.05 *vs*. NC. n = 6. (C) The proliferation curve of SGC7901/DDP after overexpression of TET2. *p < 0.05 or **p < 0.01 *vs*. NC. n = 6. (D) Representative pictures of cell apoptosis. (E) The statistical chart represents the relative cell apoptotic rate. **p < 0.01 *vs*. NC. n = 3. (F) The immunoprecipitation result represents the increased interaction of HDAC2 with TET2 (upper lane). And the western blot result represents the expression levels of IKB-ζ (middle lane). (G) The statistical chart represents the relative protein expression levels. *p < 0.05 *vs*. NC. n = 3. (H) The IL-6 levels in the culture medium of SGC7901/DDP cells after overexpression of TET2. **p < 0.01 *vs*. NC. n = 6.

plasmid markedly enhanced the expression of TET2 at mRNA and protein level. The results of cell proliferation assays and cell apoptosis analysis revealed that overexpression of TET2 attenuated the cell tolerance to cisplatin. As shown in Fig. 4C and D, overexpression of TET2 inhibited cell proliferation and promoted cell apoptosis compared with the control group. Meanwhile, overexpression of TET2 also increased the amount of HDAC2 recruited by TET2, which in turn led to a decrease in the expression of IKB-ζ (Fig. 4F). Consequently, IL-6 levels in the cell culture medium were drastically decreased in TET2-overexpressing SGC7901/DDP cells compared with the control (Fig. 4H), indicating that TET2 was negatively correlated with IL-6 level. These data collectively suggested that the enhanced TET2 expression reduced IL-6 levels and attenuated cisplatin resistance in SGC7901/DDP cells.

4. Discussion

GC is one of the most common cancers worldwide and progresses aggressively. Drug resistance is an obstacle in successful tumor treatment and it is widely accepted that the tumor microenvironment is closely associated with drug resistance. However, the underlying molecular mechanism of GC cisplatin resistance remains to be investigated with respect to the tumor microenvironment.

Cisplatin is a chemotherapeutic agent commonly used to treat a range of solid malignant tumors, including gastric, lung, breast and rectal carcinoma [5,8,17]. Although cisplatin is an effective antitumor agent, resistance to this chemotherapy medication develops in patients with GC. Increasing evidence suggests that the tumor microenvironment serves a role in the acquisition of chemical resistance [1,6], while inflammatory factors, including IL-6, are essential for the formation of the tumor microenvironment [20,23]. However, whether the tumor microenvironment, particularly the inflammatory factors, serve role in cisplatin-induced resistance of GC remains uncertain.

Increasing evidence indicates a cancer-suppressive role of TET2 in numerous malignant tumors. Nickerson et al [15] have demonstrated that decreased TET2 mRNA expression in prostate cancer is strongly associated with reduced patient survival, indicating that TET2 is a tumor suppressor gene in prostate cancer. Deng et al [3] provided evidence that low TET2 expression predicts poor overall and disease-free survival in patients with GC. In addition, reduced expression of TET2 is associated with a poor prognosis for patients with early stages of breast cancer [21]. Furthermore, a recent study indicated that TET2 recruits histone deacetylase HDAC2, therefore affecting the level of histones acetylation and the expression levels of IL-6 [22]. The documented roles of TET2 in inhibiting cancers and restraining inflammatory factor IL-6 suggest that TET2 may regulate IL-6 expression and influence the formation of the tumor microenvironment, ultimately altering drug resistance of cancer cells. In addition, we have carried out preliminary experiments investigating the expression of TETs in gastric cancer tissues and corresponding adjacent tissues, and the experimental results showed that TET2, but not TET1 or TET3 exhibited any differences in expression (data not shown). Therefore, TET2 became the focus on our research.

Based on the current understanding of TET2 in cancer progression, its association with clinical characteristics of patients with GC, and according to the role of TET2 in regulating inflammatory factor IL-6, it was hypothesized that TET2 may serve a crucial role in the development of GC drug resistance. The present study provided evidence that TET2 was negatively correlated with IL-6 in patients with GC and overexpression of TET2 markedly abrogated the tolerance to cisplatin in SGC7901/DDP cells. Furthermore, the expression of TET2 was significantly decreased in SGC7901/DDP cells compared with the non-resistant cells and loss of TET2 induced an increased expression of IL-6 potentially through histone deacetylation, which was confirmed through decreased interactions between TET2 and HDAC2.

There are several key points to be explored in future experiments. First, it remains to be investigated how cisplatin contributes to the decreased expression of TET2, as well as the crucial role of IL-6 during the development of GC cell drug resistance, which are the limitations of this study. Second, although the importance of the inflammatory factor IL-6 in the formation of the tumor microenvironment has been reported previously, it has not yet been elucidated whether other inflammatory factors are involved in the process of GC cisplatin resistance, which may be the focus of our future research. Besides, in view of the difficulty in constructing drug-resistant cells, we only performed research on one single gastric cancer cell line SGC7901/DDP, which is the limitation of our current research. Finally, *in vivo* experiments to validate the proposed scientific hypothesis should be performed, which is also a point for our future studies.

In summary, to the best of our knowledge, the results of the present study are the first to demonstrate the potential involvement of a TET2/HDAC2/IL-6 axis in the development of GC cisplatin resistance. The data suggested that targeting the TET2/HDAC2/IL-6 axis may present a novel therapeutic approach in the clinical treatment of GC drug resistance.

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Authors' contributions

KZ and DW were responsible for the design, writing and revision of the present study. HG was responsible for establishing the cisplatin-resistant SGC7901/DDP cell line. JZ, DZ and YZ performed western blot assay and immunohistochemistry. ZZ and YX contributed to the data analysis and cell culturing. YL performed RT-qPCR assays. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki. The present study was approved by the Ethical Committee of the General Hospital of Heilongjiang Province Land Reclamation Bureau (Harbin, China) and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

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