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Interference of lysine-specific demethylase 1 inhibits cellular invasion and proliferation in vivo in gastric cancer MKN-28 cells

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ABSTRACT

Background: Lysine-specific demethylase 1(LSD1), the first identified histone demethylase, plays an important role in the epigenetic regulation of gene activation and repression. Up-regulated LSD1expression has been reported in several malignant tumors.Our aim, therefore, was to better understand the mechanisms underlying the upregulation of LSD1 in gastric cancer.
Methods: We used lentiviral shRNA to knockdown LSD1 in the gastric cancer MKN-28 cell line. Cell proliferation was measured by MTT assay while cell apoptosis was assessed by Annexin V-FITC/PI double staining flow cytometry. The invasive potential of gastric cancer cells was determined by matrigel invasion assay. Protein expression was detected by Western blot. *In vivo*, the effect of knocking down LSD1 on tumor growth and protein expression in gastric cancer cells in nude mice was investigated.
Results: LSD1 knockdown in MKN-28 cell lines resulted in increasing the activity of cisplatin in vitro and the inhibition of cancer cell proliferation and invasion, and induced cell apoptosis. The expression of TGF-β1, VEGF, Bcl-2, β-catenin, p-ERK and p-Smad 2/3 proteins was inhibited in LSD1 knockdown cells. Moreover, in an *in vivo* model of gastric cancer, LSD1 knockdown suppressed tumor growth and protein expression.
Conclusion: LSD1 knockdown affected the fuction of gastric cancer MKN-28 cell line. LSD1 may be a latent target in the diagnosis and therapy of gastric cancer.

1. Introduction

Gastric cancer is a common malignant disease, with one of the highest mortality rates worldwide. Gastric cancer is the fifth most prevalent malignant disease, with an estimation of 952,000 worldwide cases annually according to the World Health Organization GLOBOCAN database. It is estimated that in 2008 there were 989,600 new cases and 738,000 deaths from stomach cancer globally, with approximately 70% of both new cases and deaths occurring in developing countries [1,2]. Gastric cancer is a multifactorial disease, arising in response to a variety of environmental, infectious, and host-related factors interacting to favor its development.

Genetic alterations are a hallmark of human cancer. In recent years, the field of cancer genomics has made significant advances in the area of cancer-associated genetic lesion identification. Lysine-specific demethylase 1 (LSD1) was the first discovered histone demethylase, which specifically removesH3K4me1/2 through a flavin adenine dinucleotide (FAD)-dependent oxidative reaction [3].

LSD1 has been found overexpressed in liver [4], gastric [5], breast [6,7], bladder, lung and colorectal cancers [8], as well as in chondrosarcoma, osteosarcoma, rhabdomyosarcoma, synovial sarcoma, Ewing's sarcoma [9], and neuroblastoma [10]; over-expression of LSD1 is a good predictor of a poor prognosis in prostate and liver cancers [4,11]. Together, these and other studies suggest thatLSD1 is linked to cancer and could be a target for drug discovery [12,13]. Despite the abundant literature on LSD1 and the many studies studying inhibition of LSD1 in cancer cells, the effect of LSD1 on the biological functions of gastric cancer has not yet been investigated. Cisplatin is one of the most effective chemo-therapeutic drugs, we investigated whether the combination of

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knock down LSD1 and cisplatin was more effective for the inhibition of cancer cell proliferation and invasion, and induced cell apoptosis in vitro.

In this paper, we used LSD1 knockdown by lentiviral shRNA to study the role of LSD1 in the biological functions of gastric cancer cells, both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Cell lines

The human gastric cancer cell lines MKN-28 was purchased from the American Type Culture Collection(ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 Modified Medium (30-2001; ATCC)containing 10% FBS supplemented with 10 μg/mL bovineinsulin (Sigma-Aldrich, St Louis, Mo, USA). Cell lines were cultured in a humidified atmosphere containing 5% carbondioxide (CO_2) and 95% air at 37 °C. All media and FBS were purchased from HyClone; Logan, UT, USA.

2.2. LSD1 knockdown by lentiviral short-hairpin RNA (shRNA)

The vector, pCMV-G&NR-U6, contains separate GFP and shRNA expression elements, as well as elements required for lentiviral packaging [14]. The three target sequences of LSD1 for constructing lentiviral shRNA are as follows:

LSD1-shRNA1: ACCGGATGACTTCTCAAGA (616 bp);
LSD1-shRNA2: CAGGCATTGGAAGTTGTCA (1299 bp);
LSD1-shRNA3: GCAGAAGGCCTAGACATTA (1806 bp)

Virus packaging was performed by transient transfection of 293T cells with a transfer plasmid. Seventy-two hours after transfection, lentiviral particles were collected and filtered, and then concentrated by ultracentrifugation at 50,000*g* for 2.5 h at 4 °C [14]. Subsequently, we infected MKN-28, SGC-7901 and AGS cell lines with the lentivirus (shLSD1) in 24-well plates. Four days after infection, the knockdown efficiency of LSD1 was examined by real-time PCR.

2.3. Real-time quantitative RT-PCR (qRT-PCR)

All PCR kits were purchased from Takara (Tokyo, Japan). Total RNA was isolated from cultured cells using an RNA isoPlus kit (1 mL per 5×10^6 cells). The concentration and purity of RNA were determined by an ultraviolet spectrometer. The generation of cDNA was according to the RNA reverse transcription (RT) kit instructions.

LSD1: primer F 5′- AAGCAGGAGGACTTCAAGAC-3′,
primer R 5′-GCAGTGTGCGGTTTCTAATG- 3′.
GAPDH: primer F5′-CACCCACTCCTCCACCTTTG-3′,
primer R5′- CCACCACCCTGTTGCTGTAG-3′.

PCR conditions were: 95 °C for 15 min; 30 cycles of 94 °Cfor 30 s, 55 °C for 30 s, and 72 °C for 2 min; and 4 °Chold. Ten microliters of PCR product was analyzed on2% agarose gels. SYBR® Premix Ex Taq™ (Perfect Real Time) was used for real-time PCR (qPCR) of LSD1. The Light Cycler PCR system (Roche Diagnostics; Mannheim, Germany) was used for qPCR amplification and threshold cycle (Ct) detection. Thermal cycling conditions comprised an initial denaturation step at 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 61 °C for 30 s. Melting curves were analyzed after amplification. Each PCR reaction was done in triplicate. Relative changes in expression were calculated using the 2-ΔΔCt method, where ΔCt is the difference in threshold cycles for the target gene and reference (ACTB), and ΔΔCt is the difference between the ΔCts of the treated sample and control or calibrator. Thus, expression levels were reported as fold changes relative to the calibrator. Values were used to plot the expression of apoptotic genes using the formula, 2-ΔΔCt.

2.4. MTT proliferation assay

Cell proliferation was measured using the methyl-thiazolyl-tetrazolium (MTT) assay (Sigma) according to manufacturer's instructions. Briefly, about 1×10^4 cells were seeded in 96-well plates and cultured in RPMI-1640 supplemented with 10% FBS. Cell proliferation was examined on 0, 12, 24 and 48 h and was determined by adding MTT (5 mg/mL) and incubating the cells at 37 °C for a further 4 h. Precipitate was solubilized by the addition of 150 μL/well dimethyl sulfoxide(Sigma) and plates shaken for 10 min. The absorbance at a wave length of 490 nm was measured in each well with a microplatereader (ELx800; BioTek; Winooski, VT, USA). All experiments were repeated three times.

2.5. Determination of an optimal cisplatin concentration

The concentration of cisplatin (WA2A1211085; Qiu Pharmaceutical; Jinan, China) used during screening was determined by MTT assay. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well in RPMI-1640 supplemented with 10% FBS. After 24 h, 2,5 or 10 mol/L concentrations of cisplatin(in PBS) were added and cells cultured for 0, 12, 24 and 48 h before the determination of cell proliferation by MTT assay.

2.6. Apoptosis assays

Cellular apoptosis was quantified by FACS using an Annexin V-FITC/PI Staining kit from Biovision (Milpitas, CA, USA). Ten thousand events per sample were counted and analyzed by an Accuri C6flow cytometer (BD Biosciences; San Jose, CA, USA). Early and late apoptotic cells were identified by the localization of Annexin V and PI.

2.7. Cell cycle analysis

All cells, seeded in asix-well plate at a density of 15×10^5 cells per well, were detached using trypsin and fixed with ice-cold 70% ethanol. Cells weresubsequently stained for cell cycle analysis using a Coulter DNA PrepReagents kit (Beckman Coulter; Fullerton, CA, USA). Cellular DNA content from each sample was determined with a FacScanapparatus (Beckman Coulter). All experiments were performed in triplicate.

2.8. Invasion assays

Transwell 24-well plates coated with diluted Matrigel were used. In the upper chamber, medium was supplemented with 1% heat-inactivated FBS. In the lower chamber, 20% FBS was used as a chemoattractant. Cells were added to the upper chamber. After 48 h, medium was removed and chambers were washed twice with PBS. Non-invading cells were removed from the upper surface of the membrane by scrubbing with a cotton-tipped swab, and any invading cells were fixed with methanol for 15 min. The chambers were then stained with 0.1% crystal violet for 30 min. Six fields for each chamber were photographed using an inverted microscope and camera, and invading cells counted in each field.

2.9. Western blotting

Total proteins (30 mg/sample) from cell cultures were separated by sodium dodecyl sulfate polyacrylamidegel electrophoresis