Role of OCT4 in cisplatin treatment of testicular embryonal carcinoma

Qin Le1,2, Jie Lin1,2, Xiaoxiao Xie3, Xiangbo Yu2, Yang Cat4, Yangping Shentu5, Aihua Zhang6, Aiwu Li7

1Department of Pediatric Surgery, Qilu Hospital, Shandong University, Jinan, 250012, 2Department of Pediatric Surgery, Wenzhou 325027, 3Department of Urology, Qingtian People’s Hospital, Qingtian 323000, 4Wenzhou Medical University, Medical Function Center, Wenzhou 325000, 5Animal Experiment Center of Scientific Research, Department of Pediatric Surgery, Qilu Hospital, Shandong University, Jinan 250012, China

*For correspondence: Email: llaiwuxwymed@163.com

Sent for review: 3 April 2018  Revised accepted: 27 June 2018

Abstract

Purpose: To determine the role of embryonal transcription factor OCT4 in cisplatin treatment of testicular embryonal carcinoma.

Methods: In vitro assays were employed to assess the effect of cisplatin treatment on testicular embryonal carcinoma cell lines under OCT4 silencing. Following treatment with 500 ng/μL cisplatin, MTT assay was used to examine cell proliferation of 2012-EP and 833K-E cells with or without OCT silencing, while wound healing assay was used to examine cell migration ability. Transwell assay and crystal violet staining were employed to measure cell invasive capacity, whereas the distribution pattern of cell cycle was assessed by flow cytometry. The expression levels of several critical components in tumorigenesis related pathways with or without OCT silencing were determined by Western-blot analysis.

Results: Cisplatin enhanced OCT4-silenced cell viability at all concentration (p < 0.01) when compared to control cells. Upon treatment with 500 ng/μL cisplatin, OCT4-silenced cells showed 2- to 3-fold enhancement in cell proliferation (p < 0.001), 2-fold increase in cell migration capacity (p < 0.001), and about 1.5-fold enhancement in invasive capacity (p < 0.001) when compared to control cells. In addition, OCT4 silencing upregulated the expression level of the proteins involved in cell proliferation, cell mobility, cancer metastasis and cell cycle control.

Conclusion: The results suggest that OCT4 may serve as a therapeutic target for testicular embryonal carcinoma treatment in combination with cisplatin by modulating OCT4 expression level. This physiological evidence indicates that OCT4 downregulation contributes to cisplatin resistance in chemotherapy and subsequent disease relapse.

Keywords: OCT4, Cisplatin resistance, Testicular embryonal carcinoma, Chemotherapy

INTRODUCTION

Testicular cancer is the foremost causal factor for cancer-related mortality in adolescents and men aged 15 to 40 years worldwide [1,2], and extensive effort has been devoted to developing the most effective therapeutics [1]. Currently, cisplatin-based chemotherapy combined with surgical resection (if feasible) is the most common strategy for treating testicular...
embryonal carcinoma [3]. Although this strategy achieves high cure rates, many patients show resistance to cisplatin-based chemotherapy, which results in very poor prognosis [4,5].

The OCT4 gene is an important embryonal transcription factor that functions in stem cell pluripotency maintenance, and the expression level of OCT4 is precisely regulated [6]. The expression of OCT4 is a characteristic feature of testicular germ cell tumors. It has been found that OCT4 downregulation is involved in cisplatin resistance of testicular embryonal carcinoma [4,7]. Treatment of testicular embryonal carcinoma cells with cisplatin [8] or hypoxia [9] can result in OCT4 downregulation. Downregulation of OCT4 reduces the levels of Noxa, Puma, and microRNAs (e.g., miR17/106b), and increases the level of the cyclin-dependent kinase inhibitor p21 [10]. This reduces apoptosis in testicular embryonal carcinoma. Using immunohistochemistry, Mueller et al [4] did not observe any OCT4 expression in the residual cancer cells after cisplatin-based chemotherapy. Therefore, cancer cells lacking OCT4 or with low OCT4 expression levels may be the cause of cisplatin resistance and may be responsible for cisplatin-resistant tumor growth after relapse. However, the contribution and detailed molecular mechanism of OCT4 function in cisplatin resistance has not been fully determined [5]. The aim of this study, is to establish the relationship, if any, between OCT4 and cisplatin treatment in testicular carcinoma cell lines using in vitro assays. The results expected may throw some light on the molecular mechanisms underlying cisplatin resistance in chemotherapy.

EXPERIMENTAL

Cell culture and transfection

Human testicular germ tumor cell lines (2102EP, 833K-E, GCT 27, GCT 35, and GCT 48, Sigma-Aldrich, USA) were maintained in DMEM medium (Sigma, St. Louis, USA) with 10% fetal bovine serum (Hyclone, Utah, USA) at 37 °C in a humidified atmosphere of 95% O2 and 5% CO2. Cells were grown to 80-90% confluence, and then subjected to hypoxia conditions with about 0.1% O2 and 5% CO2 in serum starvation medium (1% FBS) for 4 hours. Then, the cells were reoxygenated by gassing with 95% O2 and 5% CO2 for 5 min.

Small interfering RNAs (siRNA) against OCT4 were purchased from Genecopoeia (Guangzhou, China). For transfection, 1 x 10^5 cells per well were seeded in 6-well plates. Cells were then transfected via Lipofectamine 2000 (Invitrogen, USA).

RT-PCR

Total RNA was extracted from human testicular germ tumor cell lines using TRIzol (Invitrogen, CA) and a Ribolyser cell disruptor (Obio, Carlsbad, CA). First-strand cDNA was synthesized from total RNA using a Reverse Transcription System kit (Promega, Madison, WI), and then diluted. The PCR program was as follows: 94 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec. The resulting cDNA was then subjected to real-time PCR amplification with SYBR Green using a StepOne Plus Real-Time PCR system (Applied Biosystems, NY). The following primers were used:

OCT4-forward, 5'-TGGTCCGAGTGTTCTGTGAA-3';
OCT4-reverse, 5'-TGTGCTAGTCGCTGCTTGAT-3'.

Western blot analysis

Protein samples were prepared from testicular embryonal carcinoma cells with RIPA buffer. SDS-PAGE gels (4 - 20 %) were adapted for isolating proteins via electrophoresis, and the products were transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk/TBST, and then incubated with primary antibodies at 4 °C overnight. Membranes were then washed and incubated with secondary antibody (1:5,000) for 1 hour, then the protein signals on the membranes were detected using enhanced chemiluminescence system. The primary antibodies used are as follows: anti-GAPDH, anti-OCT4, anti-β-catenin, anti-PCNA, anti-CDK1, anti-MMP-2, anti-MMP-9, anti-TCF, anti-NFAT, anti-BAX, anti-Bcl-2, and anti-TWIST1. The primary antibodies were all purchased from Santa Cruz (CA, USA). The working dilution for the primary antibodies was 1:1,000 except for anti-GAPDH, which was 1:2,000.

MTT assay and drug treatment

Cell viability was tested with MTT assay. The 2102EP and 833K-E cell lines were plated in 96-well microtiter plates at 3 - 5 x 10^3 cells/well. Quintuplicate wells were prepared for each condition. At 48 h after transfection, cells were washed with PBS and incubated in MTT (1 g/L) containing medium for 4 h at 37 °C. Unconverted MTT was removed (MTT Cell Proliferation Assay Kit, BioVision, Inc.), and the optical density
values were measured using the ELISA Multiskan reader at 490 nm (Thermo Fisher Scientific).

Drug sensitivity assays were conducted as described previously [11]. Exponentially growing 2102EP and 833K-E cells were seeded at 40 - 60 % confluence, and then transfected with OCT4 siRNA the next day. After 24 hours, the cells were reseeded at 3 – 5 × 10³ cells/well and allowed to adhere overnight. The cells were treated with different concentrations of cisplatin (from 0 to 750 ng/μL) for an additional 72 h. Cisplatin sensitivity was measured with the MTT assay, and the IC₅₀ of selected cells was determined after 24 h.

Flow cytometry

FACS was used to examine the cell cycle distribution. Cells were harvested and centrifuged at 500 × g for 5 min at 4 °C, and the supernatant was aspirated overnight. Cells were collected, washed with PBS, incubated in PBS for 10 min at 37 °C, and stained with propidium iodide (PI) staining buffer (50 μL of PI and 2 μL of RNase A). The staining was measured using the imaging flow cytometer FlowSight (Amnis, EMD Millipore).

Wound-healing and Transwell assays

Cell migration was explored by performing a wound-healing assay as described previously [12]. The transwell assay was performed as described [13] to assess cell invasion. Cells in the lower wells of the transwell chambers were stained with 0.2 % crystal violet, followed by imaging and counting.

Statistical analysis

Experiments were repeated at least three times, and the results were displayed as mean ± standard deviation (SD). Statistical significance was determined with two-tailed Student's t-test, Kaplan-Meier method, and log-rank test. Statistical analyses were performed with SPSS software v 16.0 (SPSS, Inc, Chicago, IL, USA) and GraphPad Prism 6.0 (CA, USA). Significance was defined as p < 0.05.

RESULTS

Expression levels of OCT4 in different testicular carcinoma cell lines

As shown in Figure 1 A, different OCT4 protein expression levels were observed in different cell lines. From these cell lines, mRNAs were extracted and subjected to RT-PCR analysis using specific primers. Relative mRNA expression levels of OCT4 are shown in Figure 1 B and C. These combined results indicated that 2102EP and 833K-E displayed relatively high expression levels of OCT4. These cell lines were selected for further experiments.

Figure 1: Expression level of OCT4 in different testicular carcinoma cell lines. 2102EP, 833K-E, GCT 48, GCT 27, and GCT 35 testicular carcinoma cell lines were tested. (A) Whole cell lysates of indicated cell lines were subjected to SDS-PAGE and western blot analyses. Relative protein levels of OCT4 and GAPDH are shown. (B) The mRNAs of the cell lines were extracted and subjected to RT-PCR analysis with specific primers. Relative mRNA expression levels of OCT4 are shown

OCT4 knockdown

As shown in Figure 2, both protein level (Figure 2 A) and mRNA level (Figure 2 B) of OCT4 were largely reduced in OCT4-silenced cells compared to control cells (p < 0.001).

Cisplatin dosage

The data for 24 h after treatment is shown as representative of the results (Figure 3 A to C). The results indicate that OCT4-silenced cells have greater viability after treatment with any of the tested concentrations of cisplatin compared to control cells (p < 0.01). The half-maximal inhibitory concentration of cisplatin were determined in OCT4-silenced cells and control cells. As shown in Figure 3 B & C, less cisplatin was needed for OCT4-silenced cells to achieve 50 % cell viability when compared to the control group (p < 0.001). Therefore, the concentration of 500 ng/μL cisplatin was chosen for further study.
Figure 2: Knockdown of OCT4 in testicular embryonal carcinoma cells. (A) Whole cell lysates of siRNA-transfected (si-OCT4) cells and non-transfected cells (control) of 2102EP and 833K-E were subjected to SDS-PAGE and western blot analyses. Relative protein levels of OCT4 and GAPDH are shown. (B & C) The mRNA extracts of the indicated cells were prepared and subjected to RT-PCR analysis. Relative mRNA expression level of OCT4 is shown; ***p < 0.001 relative to the control group.

Figure 3: Determination of cisplatin dosage for in vitro assays. (A-B) The 2102EP or 833K-E cells with or without OCT4 silencing were treated with different concentrations of cisplatin. The apoptotic marker Apo2.7 was used to differentiate viable and apoptotic cells. Cell counting was carried out at 0, 24, 48, and 72 h after treatment. Data from the 24 h after treatment time point is shown as representative of the results. (C) The IC50 of cisplatin after 24 h treatment was determined in the indicated cells; **p < 0.01 relative to the control group; ***p < 0.001 relative to the control group.

Figure 4: Tumor cell proliferation capacity under cisplatin treatment. (A & B) Cells were transfected with si-OCT4, and cell proliferation was measured with the MTT assay at 0, 24, 48, and 72 h after treatment with 500 ng/μL cisplatin; ***p < 0.001 relative to the control group.

OCT4 negatively regulated tumor cell proliferation under cisplatin treatment

Cisplatin is an important and widely used chemotherapeutic drug for testicular tumors. To investigate the function of OCT4 in cisplatin treatment, several cancer-related processes were examined. First, cell proliferation in OCT4-silenced cells treated with 500 ng/μL cisplatin was measured. As shown in Figure 4, in both 2012-EP and 833K-E cell lines, silencing of OCT4 promoted cell proliferation (p < 0.001) under cisplatin treatment. This suggests that OCT4 expression is negatively correlated with cell proliferation.

OCT4 negatively regulated tumor cell migration and cell invasion following cisplatin treatment

Next, to assess the function of OCT4 in cell migration and cell invasion in response to treatment with 500 ng/μL cisplatin, and examine tumor cell migration, wound-healing assay was performed. As shown in Figure 5 A and B, wounds closed after 48 h in OCT4-silenced cells treated with cisplatin, whereas wounds remained open in control cells.

To assess cell invasion, a transwell assay and used crystal violet staining to visualize invasive cells were performed. As shown in Figure 6A, OCT4-silenced cells treated with cisplatin had
more invasive cells than the control. The ratio of invasive cells in control versus OCT4-silenced cells \((p < 0.001)\) is shown in Figure 6B.

Figure 5: Tumor cell migration capacity under cisplatin treatment. (A) The 2102EP or 833K-E cells with or without OCT4 silencing were treated with 500 ng/μL cisplatin and subjected to the wound healing assay. Images of the wound regions were taken at 0, 24, and 48 h. Representative images at 0 and 48 h are shown. \(p < 0.001\), (B) The wound opening was measured at 0, 24 and 48 h was measured and plotted; \(***p < 0.001\) relative to the control group.

Figure 6: Tumor cell invasion capacity under cisplatin treatment. (A) The 2102EP or 833K-E cells with or without OCT4 silencing were treated with 500 ng/μL cisplatin and subjected to a transwell assay. Invaded cells were visualized with crystal violet staining. \(***p < 0.001\); (B) The number of invaded cells was counted and plotted; \(***p < 0.001\) relative to the control group.

OCT4 is required for G1/S cell cycle transition under cisplatin treatment

To investigate whether OCT4 silencing altered the cell cycle of testicular embryonal carcinoma cells, flow cytometry was performed to examine the cell cycle distribution pattern of OCT4-silenced and control cells treated with 500 ng/μL cisplatin. As shown in Figure 7, in OCT4 silenced cells, the cell population in OCT4-silenced cells increased in the G1 phase and declined in S phase, indicating a delay in the G1/S transition. These results suggest that OCT4 is required for the G1/S cell cycle transition in cells treated with cisplatin.

Figure 7: Cell cycle analysis in 2102EP or 833K-E cells treated with cisplatin. (A) to D) Cell cycle distribution was assessed using FACS. The number of cells at each cycle stage was calculated; \(***p < 0.001\) relative to the control group.

Expression level of β-catenin is positively correlated with that of OCT4

As shown in Figure 8, both the protein (Figure 8A) and mRNA (Figure 8B) levels of β-catenin were significantly reduced in OCT4-silenced cells \((p < 0.001)\), indicating that β-catenin expression is positively correlated with OCT4 expression.

Figure 8: Expression levels of β-catenin in 2102-EP and 833K-E cells with or without OCT4 silencing. (A) Whole cell lysates of the indicated cells were subjected to SDS-PAGE and western blot analyses. Relative protein levels of OCT4 and GAPDH are shown. (B & C) The mRNA extracts of the indicated cells were prepared and subjected to RT-PCR analysis. Relative mRNA expression levels of OCT4 are shown; \(***p < 0.001\) relative to the control group.

Expression levels of critical components in tumorigenicity-related pathways negatively correlate with OCT4 expression

The protein levels of critical components in
tumorigenicity-related pathways were determined by Western-blot analysis (Figure 9). Compared to the levels of the loading control GAPDH, the expression levels of PCNA, CDK1, MMP-2, MMP-9, TCF, and NFAT increased in OCT4-silenced cells, whereas the expression levels of BAX, BCL-2, and TWIST1 remained unchanged.

![Expression levels of critical components in tumorigenicity related pathways negatively correlate with OCT4 expression. Levels of selected proteins in the indicated cells were assessed using western blot analysis.](image)

**DISCUSSION**

Up to 95% of all primary testicular tumors are germ cell tumors, which are composed of seminoma and non-seminoma tumors. Testicular embryonal carcinoma is a non-seminoma tumor with an embryonic lineage [1]. Resistance of testicular germ cell tumors to cisplatin-based chemotherapy is responsible for the poor prognosis of the subgroup of patients bearing this kind of tumor. The expression level of OCT4 has been closely related to cisplatin resistance of testicular germ cell tumors [4,7]. To define the effect of OCT4 expression in testicular germ cell tumors treated with cisplatin, tumorigenicity related characteristics in OCT4-silenced testicular embryonal carcinomas treated with cisplatin were examined.

Consistent with previous findings [4], greater cell survival rate of the OCT4-silenced testicular embryonal carcinoma cells treated with all concentration of cisplatin was observed comparing to control cells. This indicates that OCT4-silenced cells display cisplatin resistance. We examined the tumorigenicity related characteristics of the OCT4-silenced cells treated with cisplatin, and observed enhancement of cell proliferation, cell migration, and cell invasion compared to controls. Taken together, these results provide physiological evidence that OCT4 downregulation contributes to cisplatin resistance during chemotherapy and disease relapse.

Activation of the Wnt/β-catenin signaling pathway is one of the most important and well-investigated pathways in many tumor types, and β-catenin is responsible for the transcription initiation of various target genes [14]. OCT4 is a direct target of β-catenin/TCF-mediated transcription in undifferentiated embryonic stem cells [15]. The results showed that OCT4 silencing reduced β-catenin expression levels, suggesting that a feedback loop might exist between these two proteins. However, a clear increase in TCF protein expression levels were subsequently examined. The β-catenin and TCF complex functions in initiating target gene transcription. Future studies should perform a detailed examination of β-catenin/TCF and other Wnt/β-catenin signaling pathway components (e.g., protein localization, stability, and posttranslational modification).

The levels of several critical proteins involved in tumorigenicity related events were examined. Compared to control tumor cells, OCT4-silenced tumor cells had greater viability under cisplatin treatment, demonstrating an anti-apoptosis effect. In agreement, no changes in the levels of Bcl-2 and Bax proteins, which are critical regulatory factors in initiating apoptosis [16,17], were observed. PCNA participates in DNA replication and serves as a marker for cell proliferation [18]. PCNA expression levels increased in OCT4-silenced testicular tumor cells, consistent with enhanced cell proliferation. CDK1 is a cell cycle regulator, and abnormal CDK1 function is crucial tumorigenic events [19]. Accumulation of CDK1 is associated with cancer growth [20,21]. Enhanced protein levels of CDK1 were consistently observed in OCT4-silenced cells, suggesting that these cells had enhanced cell proliferation comparing to control cells. MMP-2 and MMP-9 are two matrix metalloproteinase family members. They function in breaking down the extracellular matrix and in cancer metastasis [22]. The NFAT is a transcription factor involved in breast cancer metastasis [23]. Enhanced levels of MMP-2, MMP-9, and NFAT proteins were detected, which is consistent with enhanced cell migration and invasion in OCT4-silenced cells treated with cisplatin. The level of TWIST1 (a zinc finger transcription factor involved in different phases of tumorigenicity) was examined because TWIST was reported to have a critical role in lung cancer cell chemoresistance to cisplatin [24]. However, the level of TWIST1 protein remained unchanged in OCT4-silenced testicular embryonal carcinoma cells.
cells compared to control cells, which suggested that cisplatin resistance in OCT4-silenced cells was unrelated to TWIST1. The expression levels of the above-mentioned proteins also should be examined under cisplatin treatment.

CONCLUSION

It is here proposed that OCT4 may serve as a therapeutic target for the treatment of testicular embryonal carcinoma in a combination therapy with cisplatin by modulating OCT4 expression level.

DECLARATIONS

Acknowledgement

This work was funded as part of a project titled ‘Construction of Overexpression OCT4 Gene Mouse Model and Its Role in Testicular Injury’ (grant no. Y20170191).

Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Aihua Zhang and Aiwu Li designed all the experiments and revised the paper. Qin Le, Jie Lin, Xiaoxiao Xie and Xiangbo Yu performed the experiments, Qin Le, Jie Lin, Yang Cai and Yangping Shentu wrote the paper. Qin Le and Jie Lin contributed equally to the work.

REFERENCES

2. Eble JN, Sauter G, Epstein JI, Sesterhenn IA (Eds), WHO Classification of Tumours. Pathology and Genetics. Tumours of the Urinary System and Male Genital Organs Ch. 4, 250-262.


