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CRISPR/Cas9-mediated knockout of Lcn2 effectively enhanced CDDP-induced apoptosis and reduced cell migration capacity of PC3 cells

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ABSTRACT

Aims: Lipocalin 2 (Lcn2/NGAL) belongs to lipocalin superfamily with diverse functions. The precise function of Lcn2, particularly in cancer development, remains to be elucidated yet. In an attempt to knockout of Lcn2 expression by CRISPR/Cas 9 technology in a highly aggressive and invasive prostate cancer cell line and to evaluate the combination therapy with cisplatin (CDDP), this study was conducted.

Main methods: Control CRISPR/Cas9 plasmid and homology-directed repair plasmid or validated human Lcn2 CRISPR/Cas9 KO plasmids were co-transfected into PC3 cells using fugene HD transfection reagent. The stable cells were selected in the presence of puromycin. Correspondingly, knock out of Lcn2 was evaluated by RT-PCR, ELISA, and immunocytochemistry. PC3-Scr (control) and Lcn2-KO (PC3 cells in which lcn2 has been knocked out) were treated with or without cisplatin (CDDP). Cell proliferative ability was measured by WST-1 and colony-formation assays. Apoptosis was evaluated by DAPI staining, *in situ* cell death detection (TUNEL) assay, and cell death detection ELISA plus methods. The migration capabilities were studied by wound healing/scratch and transwell assays.

Key findings: Lcn2 knock out in a highly aggressive and invasive cancer cell like PC3 decreased cell proliferation and increased the sensitivity of CDDP. Conspicuously, loss of Lcn2 expression effectively enhanced CDDP-induced apoptosis in PC3 cells. Lcn2 knock out by CRISPR/Cas9 technology decreased the cell migration capacity of PC3 cells as well.

Significance: Lcn2 not only is a valuable and useful biomarker for diagnosis and prognosis of prostate cancer but also and more importantly is a potential novel emerging therapeutic target.

1. Introduction

Prostate cancer (PCa) is the most common and one of the deadliest cancers among men worldwide [5]. There are a wide range of treatments for PCa including surgery, chemotherapy, radiotherapy, *etc.* [1]. Although, in most cases, these treatments are effective and the patients become cancer-free, but they are far from 100% effective and also 100% of the time. Additionally, each of these approaches has many side effects and complications left behind (*e.g.* incontinence, sexual problems *etc.*) [1]. In order to overcome these gaps, expanding our insight about the disease and its underlying mechanisms and designing novel treatment options seems absolutely essential.

Given the fact that the cancer essentially has a genetic basis and many genes are reportedly up−/down-regulated during the disease [13,30], identifying and targeting the deregulated genes might be useful for both enhancing our understanding of the disease and improved cancer therapy. Lipocalin-2 (*Lcn2*) is one of the genes that are upregulated in most types of cancer [8,24,34].

Lcn2 is a small and secretory protein which belongs to lipocalins superfamily [7]. Physiologically, Lcn2 has a wide range of roles in the body, including anti-bacterial [4,12], anti-oxidant [2,3,14,25–28,31], pro- or anti-apoptotic [18], iron transporter [33], *etc.* Furthermore, from a pathophysiological point of view, a bulk body of studies has reported that Lcn2 is upregulated and involved in the development of

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almost all types of cancer [8,24,34]. Moreover, recently it has been reported that Lcn2 plays an important role in resistance of cancer cells against some common anti-cancer drugs, a phenomenon that is also known as "chemoresistance" [20,23,25,35]. However, it is worthy to mention that there are few papers dealing with the tumor suppressor role of Lcn2 as well. Indeed, the relation between Lcn2 and cancer progression is organ type-specific *i.e.* in some type of cancers such as PCa and breast cancer [32], *Lcn2* acts like a proto-oncogene but in some other types such as colon cancer [19] *Lcn2* plays as a tumor suppressor factor. Considerably, as mentioned above, it has been well-known that Lcn2 is upregulated in PCa. Hence, it's targeting by a powerful and effective method could be a promising strategy to suppress PCa progression. In order to genetically silence a gene's expression, there are three common methods available today; 1. Zinc finger nucleases (ZFNs), 2. transcription activator like effector nucleases (TALENs), and 3.clustered randomly interspersed short palindromic repeats (CRISPR)/CRISPR associated-9 (Cas9) [11]. Among these techniques, CRISPR/Cas9 is a genetic engineering-based method that is considerably simpler, cheaper and stronger than the others. CRISPR/Cas9 is one of the novels, commercially available and powerful genetic engineering tools that has made "gene knockout" processes more efficient, cost-effective and easier than before.

Taken all together, in the current study, we tried to target one of the crucial genes *i.e.* Lcn2 – that has been implicated in the development of a variety of cancer types [24] and even in the chemoresistance of cancer cells [23,35] – by CRISPR/Cas9 technique in a highly invasive, aggressive and hormone-refractory cell line of PCa namely PC3, followed by evaluation of this targeting in combination therapy with CDDP (the generic drug name of cisplatin) in terms of cytotoxicity, apoptosis, and cell migration capacity in an *in vitro* study.

2. Materials and methods

2.1. Cell culture

Human PCa cell line, PC3, was purchased from national cell bank, Pasteur Institute of Iran. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media with 10% fetal bovine serum (FBS) and incubated with 5% CO2 at 37 °C.

2.2. Vectors and transfection

Validated Human Lcn2 CRISPR/Cas9 KO Plasmids, and Homology-directed repair (HDR) plasmid, and Control (Scramble) CRISPR/Cas9 Plasmid (Scr) were purchased from Santa Cruz biotechnology (SCBT, USA). In this way, Lcn2 CRISPR/Cas9 KO Plasmid consists of a pool of 3 plasmids, each encoding the Cas9 nuclease and a target-specific 20 nt guide RNA (gRNA) designed for maximum knockout efficiency. The three Lcn2-specific gRNAs sequences were as; 5′-CATCCCGCTGCCTA GGGCGC-3′, 5′-AGCCGTTGCGCCCGATGCCG-3′ and 5′-CCAGTTCTCAC CGCGGCATC-3′. In this account, CRISPR/Cas9 KO Plasmids and HDR plasmids or Control CRISPR/Cas9 Plasmid were co-transfected into PC3 cells using fugene HD transfection reagent (Roche, USA) according to manufactures' protocol. Briefly, PC3 cells were seeded into a 6-well plate. After 24 h that the cells reached 60–70% confluent, the culture media were removed and the cells were washed with phosphate buffer saline (PBS), 1.2 mL of C1 solution was added to the well. At 24 h post-transfection, following removal of the C1 solution and washing the well with PBS, 1.5 mL of selection medium (complete medium containing 2 μg/mL of puromycin) were added. Successful transfection of the HD and CRISPR plasmids were visually verified under florescent microscope.

2.3. RNA Extraction and RT-PCR

Total RNA was extracted using the RNX-Plus reagent kit (Cinnaclon, Iran) from the cultivated cells according to the manufacturer's instructions. In this way, quantitative measurement was done using a Spectrophotometer NanoDrop ND-1000 (NanoDrop, USA) and also for qualitative measurement, all extracted RNAs were electrophoresed on gel agarose 1.5%. Additionally, cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, USA) and also the total RNAs were considered as the template. PCR was performed using a T100™ Thermal Cycler (Bio-Rad, USA) with *β-actin* as an internal control.

PCR condition included a primary denaturation step at 95 °C for 5 min followed by 35 cycles of PCR (95 °C for 30 s, 57 °C for 30 s, 72 °C 30 s) and 72 °C for 5 min. Lcn2 primer pairs were, forward: 5′-TCACC TCCGTCCTG TTTAGG-3′ and reverse: 5′-CGAAGTCAGCTCCTTGGT TC-3′. β-actin primer pairs were, forward: 5′-TTCTACAATGAGCTGCGTG TGC-3′ and reverse: 5′-GTGTTGAAGGTCTCAAACATGAT-3′. The PCR products were electrophoresed on 2% agarose gel.

2.4. Immunocytochemistry

Immunocytochemical (ICC) staining was performed on the cultured PC3 cells grown on sterile coverslips. The coverslips were incubated in Lcn2 antibody (abcam, UK) diluted 1:1000 at 4 °C overnight and incubated in second antibody (abcam, UK) diluted 1:500 at 37 °C for 30 min. Then the coverslips were counterstained with 4′,6-diamidino-2-phenylindole, DAPI, (Sigma-Aldrich, USA).

2.5. Enzyme Linked Immunosorbent Assay (ELISA)

Expression of Lcn2 at protein level was also measured by ELISA kit ((R&D Systems, USA) as instructed by the manufacturers. Concisely, cell culture medium of PC3-Scr and Lcn2-KO cells were collected, centrifuged at 1,800 rpm for 10 min to remove cells and debris, and were used for measurement of the proteins' concentration. The concentrations were calculated according to the standard curves created using standard samples that were provided by the kit.

2.6. Cell proliferation assay and colony formation assay

Cell proliferation was quantified using the Cell Proliferation Reagent Water Soluble Tetrazolium-1 (WST-1) solution (Sigma-Aldrich, USA) according to the manufacturer's instructions. For the colony formation assay, 1,000–2000 of PC3 or KO cells/well were incubated in RPMI 1640 medium containing 10% FBS for 2 weeks. The colonies were fixed with methanol 100% and stained with Giemsa for 20 min. The number of colonies having > 50 cells was counted using an inverted microscope and analyzed using ImageJ software.

2.7. DAPI staining

PC3-Scr or Lcn2-KO cells were seeded into 6-well plates and grown on sterile coverslips as 5 × 105 cells/well. After 24 h, the culture media were changed and the cells were incubated with 500 μg/mL of CDDP in complete medium for 6 h. At 6 h post-treatment, following discarding the culture media and washing the cells with PBS, they were fixed with methanol 100%. Apoptosis assay was performed with DAPI staining according to the manufacturer's instructions. Finally, two random images under a florescent microscope (at 10× magnification) were taken for each well. The numbers of apoptotic and non-apoptotic cells were counted and analyzed using ImageJ software.

2.8. In Situ Cell Death Detection (TUNEL) assay

PC3-Scr or Lcn2- KO cells were cultured and treated with 500 μg/mL CDDP. Detection and quantification of apoptosis at single cell level, based on labeling of DNA strand breaks (TUNEL) were performed by *in situ* cell detection kit (Roche, Germany) according to the kit instruction.

The coverslips were also counterstained with DAPI. Finally, two random images under a florescent microscope (at 10× magnification) were taken for each well. The number of apoptotic and non-apoptotic cells was counted and analyzed using ImageJ software.

2.9. Cell death detection ELISA

Apoptosis was also further studied by Cell Death Detection ELISA ^{plus} (Roche, Switzerland) which monitors DNA fragmentation. PC3-Scr or Lcn2-KO cells were seeded into 6-well plates as 5×10^5 cells/well. The cells were harvested using Trypsin-EDTA after treatment with CDDP. Cell pellets were collected and apoptosis assays were performed according to the manufacturer's instructions.

2.10. Wound healing/scratch assay

PC3-Scr or Lcn2-KO cells were seeded into 6-well plates. After 24 h, similar straight scratches have been made on monolayers in each well using 1000 µl sterile pipette tips and washed twice with culture medium. At 0 h, 24 h, 48 h and 72 h time points, two random pictures under a microscope (at 4× magnifications) were taken for each wound. The areas between the edges of the wounds have been measured and analyzed using ImageJ software.

2.11. Transwell assay

The control PC3 or Lcn2-KO cells (5×10^4 cells/well in 60 µl in RPMI) were seeded on SPL 24-well cell culture inserts (6.5 mm diameter, SPL, Korea) with 8.0 µm pores in 45 µL serum-free medium at their upper surface. RPMI with 10% fetal bovine serum was added to the lower wells to stimulate migration. Cells were further incubated for 6 h. Then, medium was discarded and migrating cells were stained with Hematoxylin/Eosin for 5 min at room temperature. The cells that had migrated to the lower surface of the filter membrane were taken photos under the microscope. Three independent experiments for each sample were carried out.

2.12. Statistical analysis

The experiments were performed in three independent tests of triplicates. Student's *t*-test and/or ANOVA were performed for statistical analyses. All values were expressed as mean ± SD and considered significantly different when p value was < 0.05.

3. Results

3.1. CRISPR/Cas9-mediated knockout of Lcn2 decreased cell proliferation

To investigate whether Lcn2 has any role in carcinogenesis of a highly aggressive PCa cell line *i.e.* PC3, Lcn2 expression was knocked out by CRISPR/Cas9 technology in PC3 cells initially. Schematic representation and overview of the study have been shown in Fig. 1. Shortly, following transfection of the PC3 cells with the CRISPR/Cas9 plasmids and culturing of the cells in the selection medium for 1 week, single cell colonies were established by serial dilution followed by confirmation assays for Lcn2 knockout (KO) and cell proliferation assays. Our pilot and initial results indicated the established Lcn2-KO colonies exhibited similar characteristics in terms of lack of Lcn2 expression and reduced proliferation (data not shown). Therefore, we mixed the three established colonies, named Lcn2-KO, and studied the effects of the Lcn2 knockout in these cells in comparison to the scramble PC3 cells (PC3-Scr). Confirmation of the Lcn2 knockout in Lcn2-KO cells firstly confirmed in mRNA level by RT-PCR (Fig. 2A) followed by immunohistochemistry (Fig. 2B) and ELISA (Fig. 2C). The results indicated the absence of Lcn2 expression in the Lcn2-KO cells. Cell proliferation assay indicated that knockout of Lcn2 in PC3 cells slowed their proliferation rate (Fig. 3A). Consistently, the colony formation assay results further confirmed the cell proliferation assay results (Fig. 3B). Taken altogether, these results suggested the oncogenic role of Lcn2 in the PCa progression.

3.2. Loss of Lcn2 expression resulted in enhancing of sensitivity of PC3 cells to the chemotherapeutic agent

A number of studies have been reported that Lcn2 is involved in chemoresistance of cancer cells against chemotherapy drugs [23,35]. To investigate whether knockout of Lcn2 would increase/decrease the cytotoxicity effects of CDDP on the cells, the Lcn2-KO and PC3-Scr cells were treated with different concentrations of CDDP followed by cell proliferation assays. Within our assumption, the absence of Lcn2 increased the cytotoxicity effects of CDDP on PC3 cells (Fig. 4). It is noteworthy that anticancer therapies including radio-, immuno- and chemo-therapy primarily exert their effects *via* activating cell death pathways including apoptosis in cancer cells. However, apoptosis-evading behavior of cancer cells results in treatment resistance, especially chemoresistance that is a major challenge for cancer therapy. Therefore, next, in order to determine whether knockout of Lcn2 could increase CDDP-induced apoptosis in the PC3 cells, the Lcn2-KO and the PC3-Scr cells were treated with CDDP (500 µg/mL) for 6 h followed by evaluation of apoptosis using DAPI staining, *In Situ* Cell Death Detection (TUNEL), and cell death detection ELISA ^{plus} methods. The TUNEL assay results revealed that CDDP induced a few apoptotic cell deaths (about 16%) in the control cells (PC3-Scr) but interestingly this reached to 47% in the Lcn2-KO cells (Fig. 5A). Consistently, the DAPI staining results indicated the CDDP treatment stimulated apoptosis in the Lcn2-KO cells nearly four times more than in the PC3-Scr cells (Fig. 5B). Finally, to further evaluate and confirm the TUNEL and DAPI staining results, we also performed a quantitative ELISA-based assay for apoptosis. Consistent with previous findings, we found that knockout of Lcn2 prominently increased CDDP-induced apoptotic cell death in the prostate cancer cell line (Fig. 5C). Altogether, these results unanimously support the idea that Lcn2 acts like a strong shield against an anti-cancer drug, CDDP, at least in part, through inhibiting of CDDP-mediated apoptotic cell death.

3.3. Targeting of Lcn2 expression decreased the cell migration capacity of PC3 cells

Migration is a key property of cancer *cells* and critical for cancer metastasis. Since PC3 cells are highly aggressive and invasive, we evaluated the migration capacity of the Lcn2-KO cells in comparison with the PC3-Scr cells finally. To achieve this, scratch and transwell assays were performed. Based on the transwell assay results, the number of PC3-Scr cells that were migrated through 8-µm filter after 6 h was about six times higher than that of Lcn2-KO cells (Fig. 6A and B). Consistently, the scratch assay experiments revealed that the PC3-Scr cells can fill the wounded area much faster than the Lcn2-KO cells at any given time point that studied for 72 h (Fig. 6 C and D). Of note, when the cells were scratched by yellow pipette tips (100 µl pipette tips), the scratch area was more filled in the control cells compared with the Lcn2-KO cells after 72 h (Supplementary Fig. 1).

Taken together, these results strongly suggest that targeting of Lcn2 expression by CRISPR/Cas9 technology in a highly invasive and aggressive prostate cancer cell line might be considered as a potential, novel, and additional modality, at least in part, for the treatment of prostate cancer cells.

4. Discussion

New therapeutic approaches in cancer have attracted a lot of attention recently. In this way, we tried to take basic and essential steps toward targeted cancer therapy owing to CRIPR/Cas9 technology in our

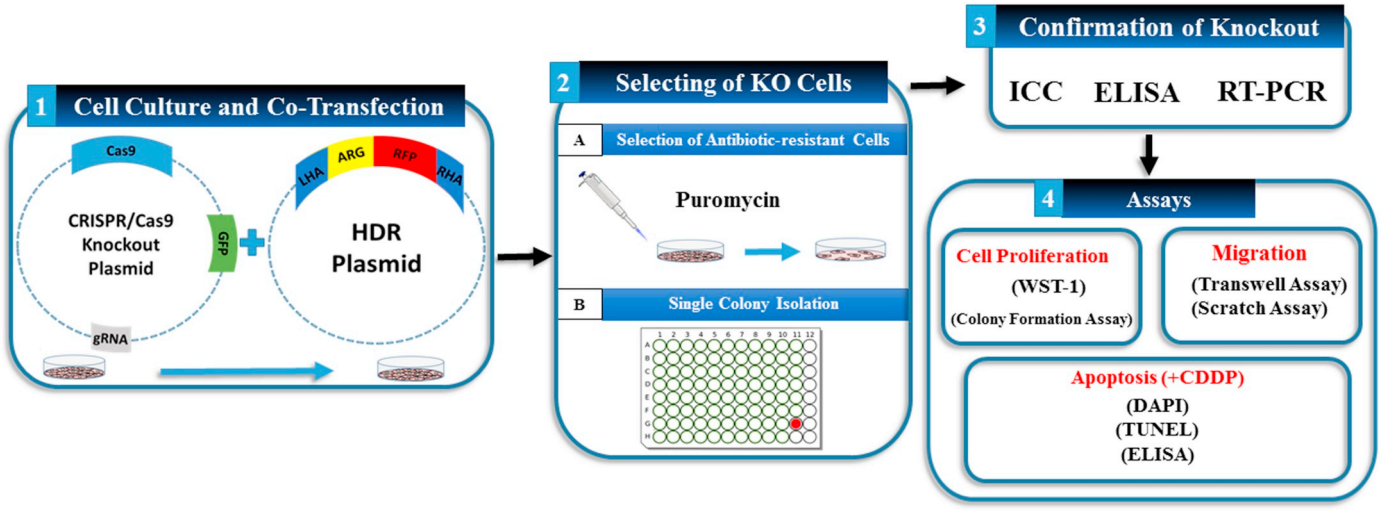
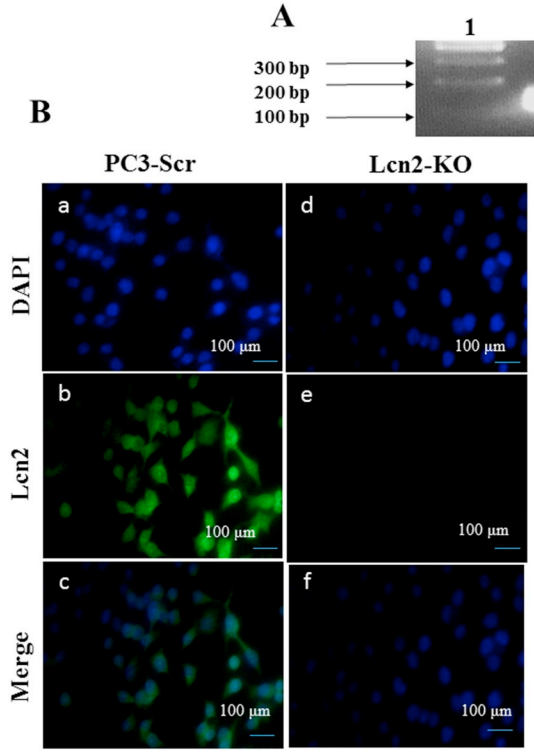


Fig. 1. Schematic Representation and Overview of the Study. This figure illustrates our workflow in the present study. Briefly: 1) In order to create an Lcn2-deficient PC3 cell line, the cells were cultured and then co-transfected with the CRISPR/Cas9 plasmids (which are verified and specifically target *Lcn2* gene in the human genome). 2) The co-transfected cells were incubated and selected in the presence of puromycin for one week. 3) Lcn2 knockout was confirmed at transcriptional (by RT-PCR) and translational levels (by ICC and ELISA). 4) Finally, effects of the Lcn2 knockout on some cellular characteristics (including chemoresistance, cell proliferation, apoptosis and migration) were assessed by their own corresponding and specific techniques as shown in the figure.

project. Inadequate knowledge and information about cancer and its underlying mechanisms have resulted in the absence of an efficient anti-cancer therapy strategy yet. Furthermore, the aims of nearly all of the current treatments are just to alleviate the consequences and complications of the disease instead of targeting the main underlying causes. Remarkably, cancer is a complex and multifactorial disease with different structural mechanisms and many studies have demonstrated that genetic dysregulations, for example, aberrant expression of certain



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Samples	OD 450
Lcn2, 10 ng/mL*	1.648 ± 0.122
Lcn2, 5 ng/mL*	0.932 ± 0.131
Lcn2, 2.5 ng/mL*	0.522 ± 0.128
Lcn2, 1.25 ng/mL*	0.310 ± 0.065
PC3-Scr	0.675 ± 0.097
Lcn2-KO	0.010 ± 0.004

*Standard concentrations of human Lcn2 was provided in the ELISA kit.

Fig. 2. Confirmation of the Lcn2 Knockout in PC3 Cells. In order to indicate that Lcn2 expression was successfully abrogated in PC3-KO cells, three confirmational assays were performed comprising RT-PCR, ICC (B) and ELISA (C). (A) For RT-PCR, both PC3-Scr and Lcn2-KO cells were cultured in T-25 flasks and after RNA extraction, equal amounts of the RNAs were used for cDNA synthesis. Finally, PCR was performed with specific primers for Lcn2 and β-actin, and PCR products were ran in 2% agarose gel. (lane1 = 100 bps ladder, lane2 = β-actin of PC3-Scr cells, lane3 = β-actin of Lcn2-KO cells, lane4 = Lcn2 of PC3-Scr cells, lane5 = Lcn2 of Lcn2-KO cells) Absence of lcn2-specific bond in lane 5 indicates that Lcn2 was successfully knocked out in Lcn2-KO cells at transcriptional level. (B) For ICC, PC3-Scr and Lcn2-KO cells were cultured on sterile coverslips in 6-well plates and following fixation, the cells were stained with lcn2-specific antibodies. DAPI staining was used for counter-staining. (Green = Lcn2, blue = nucleus) absence of any green colour in B-e and B-f indicates that lcn2 was successfully knocked out in Lcn2-KO cells at translational level. Overall, expression of Lcn2 was detected in the cells but no expression was observed in the Lcn2-KO cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

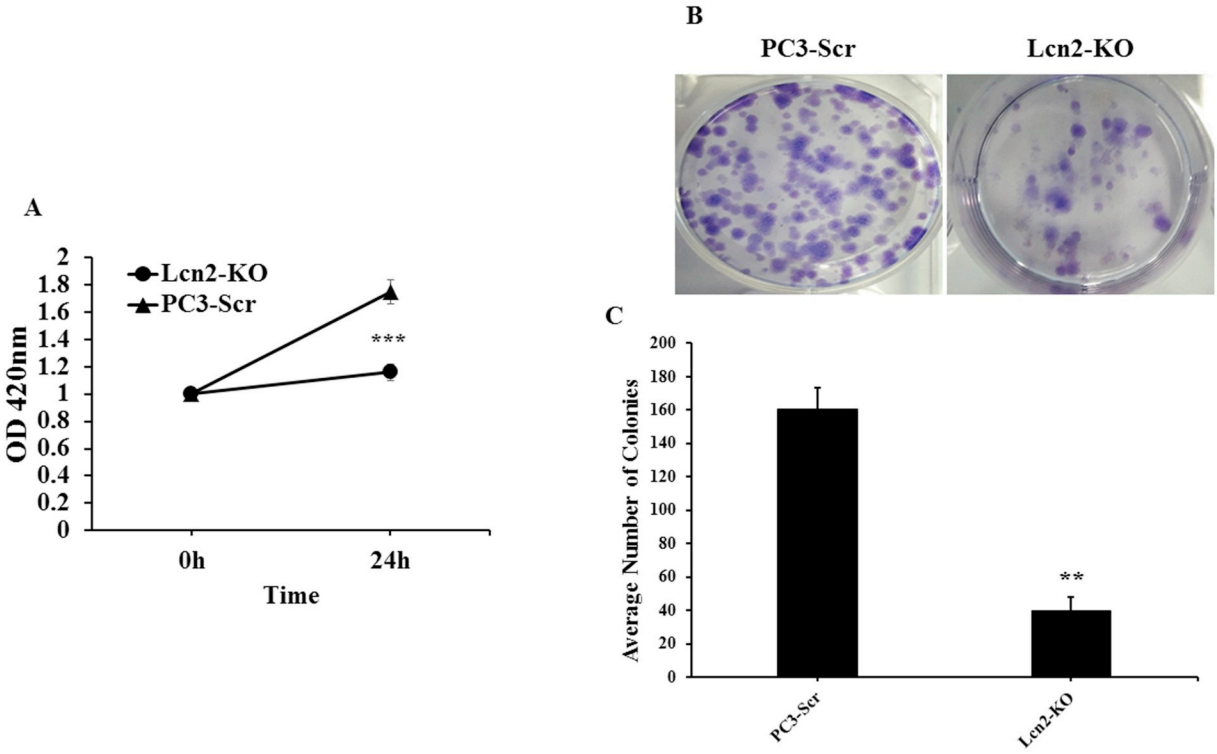

Fig. 3. CRISPR/Cas9-mediated Knockout of Lcn2 Significantly Inhibited PC3 Cells Growth. (A) For WST-1 assay, equal numbers of PC3-Scr and Lcn2-KO cells were seeded into 96-well plates and absorbance at 420 nm were measured at 0 and 24 h time points. (B and C) For colony formation assay, equal numbers of PC3-Scr and Lcn2-KO cells were seeded into 6-well plates. After 2 weeks, the cells were stained with giemsa and the colonies with > 50 cells were counted in each well. The results of both assays obviously indicated that the Lcn2 knockout remarkably attenuated PC3 cells proliferative abilities. (Mean ± SD, ***$P < .001$, **$P < .01$).

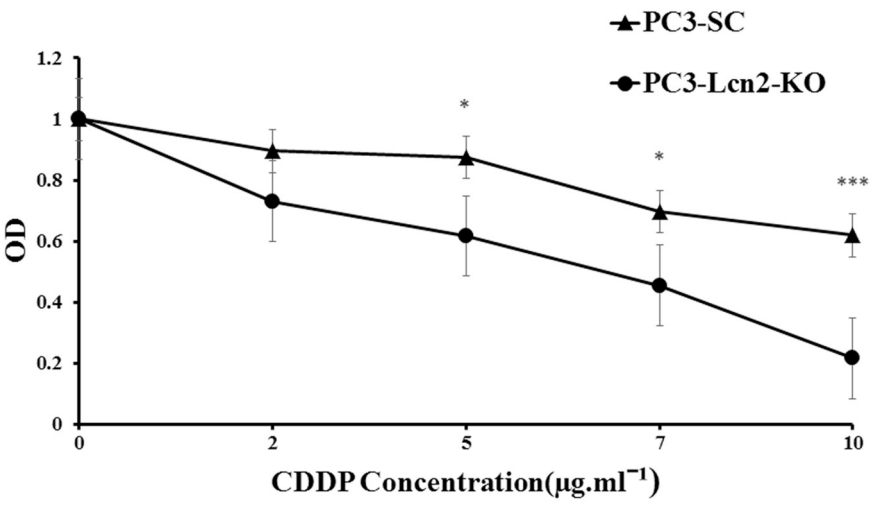

Fig. 4. Lcn2 knockout considerably increased CDDP-induced cytotoxicity. Proliferation of PC3-Scr and Lcn2-KO cells in the presence or absence of CDDP was also investigated. In this regard, equal numbers of both cells were seeded into 96-well plates and treated with different concentrations of CDDP, and quantified with WST-1 assay. The results revealed that the Lcn2 knockout significantly increased sensitivity of PC3 cells against CDDP. (Mean ± SD, ***P < .001).

genes play a major role in many types of cancer [13,30].

In this study, by CRISPR/Cas9 technique Lcn2 gene was knocked out in a highly aggressive and invasive prostate cancer cell line followed by evaluation of this targeting in combination therapy with CDDP especially in terms of induction of apoptosis.

Our results revealed that the knocking out of Lcn2 *via* CRISPR/Cas9 effectively resulted in reduced cell proliferation after 24 h. Downregulation of Lcn2 by shRNA in PC3 and DU-145 cells has been studied by Tung et al. [29]. Importantly, they did not observe any difference in proliferation between the control and those Lcn2-down-regulated cells after 24 h. The first significant difference was observed after 48 h indicating that the efficiency of knockout by CRISPR/Cas9 technique comparing to the knockdown by shRNA. Of note, the reduction of Lcn2 expression that reported by Tung *et al* was 85% whereas in our study no expression of Lcn2 was detected as investigated by RT-PCR, ELISA, and immunohistochemistry.

It is important to say that the precise mechanisms underlying Lcn2 promotes cancer growth including prostate cancer remain unknown yet. Lcn2 exerts its oncogenic effects through several main mechanisms including; 1- being an important local iron transporter, Lcn2 imports iron into cancer cells, thereby increasing intracellular iron levels, which in turn facilitates the tumor cells proliferation [6,17,22]. 2- Lcn2 elevates expression and activity of gelatinases' (matrix metalloproteinase-9 (MMP9) and matrix metalloproteinase-2 (MMP2)) which in turn renders the extracellular matrix (ECM) softer thereby cancer cells become motile and invasive [6]. 3- Lcn2 induces epithelial to mesenchymal transition (EMT) (an integral part of metastasis) in tumor cells [24], 4- Lcn2 drives angiogenesis around some primary tumors by upregulating

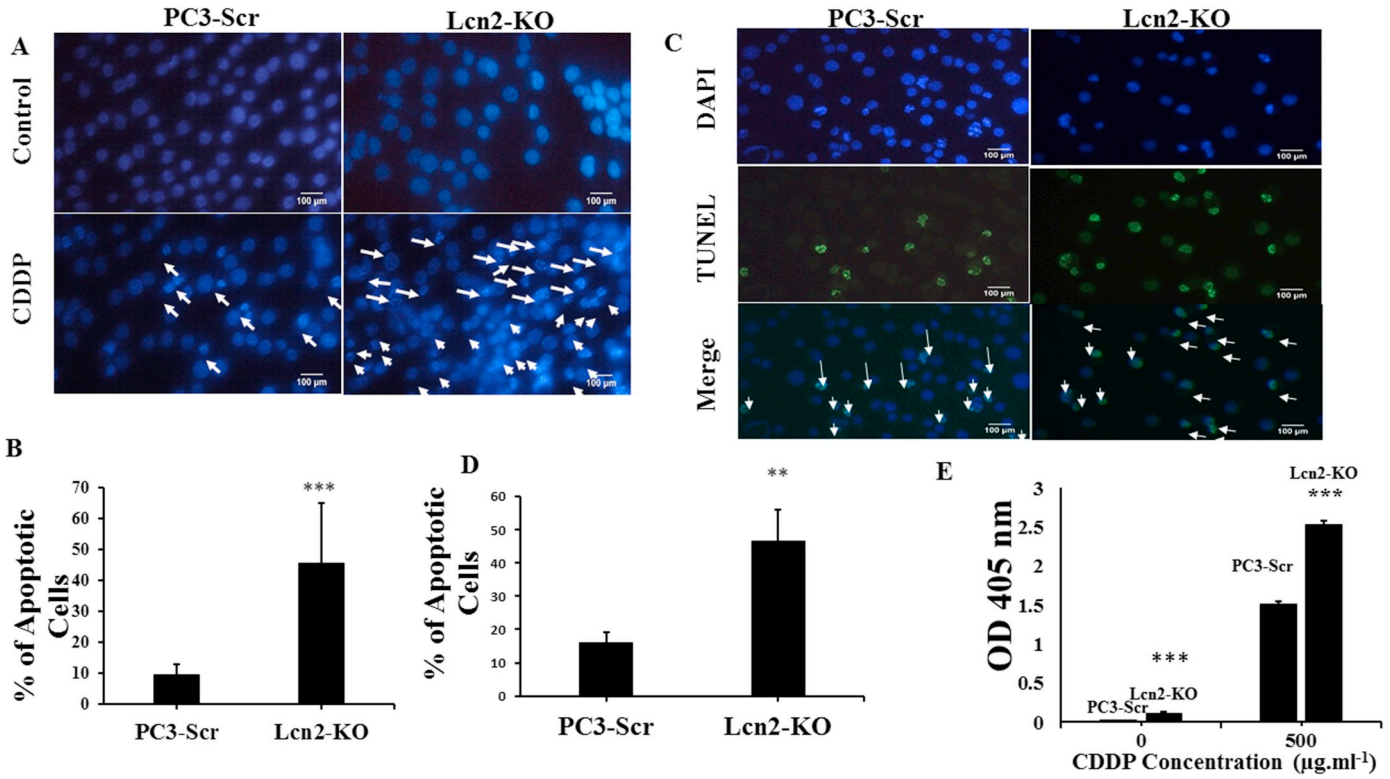

Fig. 5. Knockout of Lcn2 Significantly Increased CDDP-induced Apoptosis in PC3 Cells. For DAPI (A and B) and TUNEL (C and D) assays, equal numbers of PC3-Scr and Lcn2-KO cells were cultured on sterile coverslips in 6-well plates. Then the cells were treated with 500 µg·mL^{-1} CDDP for 6 h. After the treatment, the cells were fixed and stained with *In Situ* Cell Death Detection (TUNEL) kit according to the manufacturer's protocol. Both the DAPI staining and the TUNEL assays showed that the Lcn2-KO cells are much more sensitive to CDDP-induced apoptosis than the control cells. (E) For Cell Death Detection ELISA ^{plus} assay, equal numbers of PC3-Scr and Lcn2-KO cells were seeded into 96-well plate and treated with 500 µg·mL^{-1} for 6 h and then the protocol was carried out according to the manufacturer's manual. In consistence with above findings, Cell Death Detection ELISA ^{plus} results further supported the anti-apoptotic roles of Lcn2 against CDDP in PC3 cells. The percentage of apoptotic cells after 6 h treatment with CDDP in the Lcn2-KO cells was significantly higher than that of the PC3-Scr cells. (Mean ± SD ***$P < .001$, **$P < .01$).

and activating vascular endothelial growth factor (VEGF) in nearby normal cells [20,21], *etc.*

Tung et al. showed that down-regulation of Lcn2 up-regulates p21 and p53, induces G0/G1 cell cycle-arrest as well as down-regulation of the cell cycle- regulator cyklin D1 suggesting Lcn2 by regulating the G0/G1 phase checkpoint target cyclin D, p53 and p21 may promote the proliferation prostate cancer cells. Though, clarifying the precise mechanisms in this regards would be the subject of future study.

In 2016 Ding G and his colleagues indicated that lentivirus-mediated Lcn2 overexpression in two non-invasive PCa cell lines, C4−2B and 22RV1, promoted their proliferation, while knockdown of Lcn2 resulted in the opposite results [10]. They employed siRNA technology to knockdown Lcn2 expression and followed cells proliferation for six days. Interestingly, the first significant difference was observed after four days post-knockdown, which again highlights the fact that in order to inhibit the pro-proliferative effects of Lcn2 in tumor cells, it seems the complete loss of Lcn2 expression is essential.

We found that the loss of Lcn2 expression increased the sensitivity of PC3 cells to CDDP. This finding not only highlights the importance of the combination therapies – targeting plus chemotherapy – but also might be considered as a novel strategy to tackle the chemotherapy resistance problems and to minimize the side effects of chemotherapeutic agents such as CDDP as well. Correspondingly, our findings also revealed that the targeting of Lcn2 expression in PC3 cells *via* the CRISPR/Cas9 technology effectively increased CCDP-induced apoptosis. It is well worth to mention that one of the well-known features of cancer cells is the escaping from apoptotic cell death. The mechanisms underlying cancer cells evading from apoptosis include upregulation of anti-apoptotic signals, downregulation of pro-apoptotic signals, and abnormal cross talk of apoptosis and autophagy [15,36]. On the other hand, chemotherapeutic drugs such as CDDP induce pro-apoptotic signals in cancer cells [9]; however, cancer cells by escaping apoptosis would cause cancer resistance and lead to therapeutic failure.

It has been shown that the mechanism of cisplatin-induced apoptosis is closely associated with the capability of cisplatin to produce cellular reactive oxygen species (ROS). [16]. On the other hand, we have already shown that Lcn2 protects cells against cisplatin toxicity [25]. Moreover, we have already shown that Lcn2 acts as a scavenger of ROS [26,27]. Taken together, our results might suggest that knock out of Lcn2 in the PC3 cells, at least in part, impedes in the scavenging role of Lcn2 and causes CDDP- induced apoptosis.

Since Lcn2 upregulates in a variety of cancer types, it can be concluded that our findings might introduce a novel treatment strategy *i.e.* targeting apoptosis and might be feasible and usable for cancer therapy. Our results also revealed that knockout of Lcn2 decreased the cell migration capacity of a highly aggressive and invasive prostate cancer cell line *i.e.* PC3 Cells. Supporting this notion, Tung and *et al* evaluated the effects of Lcn2 knockdown on the prostate cancer cells' ability to invade three dimensional extracellular matrix *i.e.* matrigel [29]. They also found that Lcn2 knockdown decreased the migration and invasion capacities of PC3 cells. Furthermore, they also studied the expression and activity level of MMP-2 and MMP-9. There is a direct relationship between the gelatinases expression and activity and cancer progression. They found that the knockdown of Lcn2 in the PC3 cells decreased the expression of MMP-2 and MMP-9 at the protein levels. This finding was further confirmed by a gelatin zymography assay, in which the knockdown of Lcn2 in PC3 and DU145 cells resulted in a decreased MMP-2 and MMP-9 expression, compared to the control cells. The

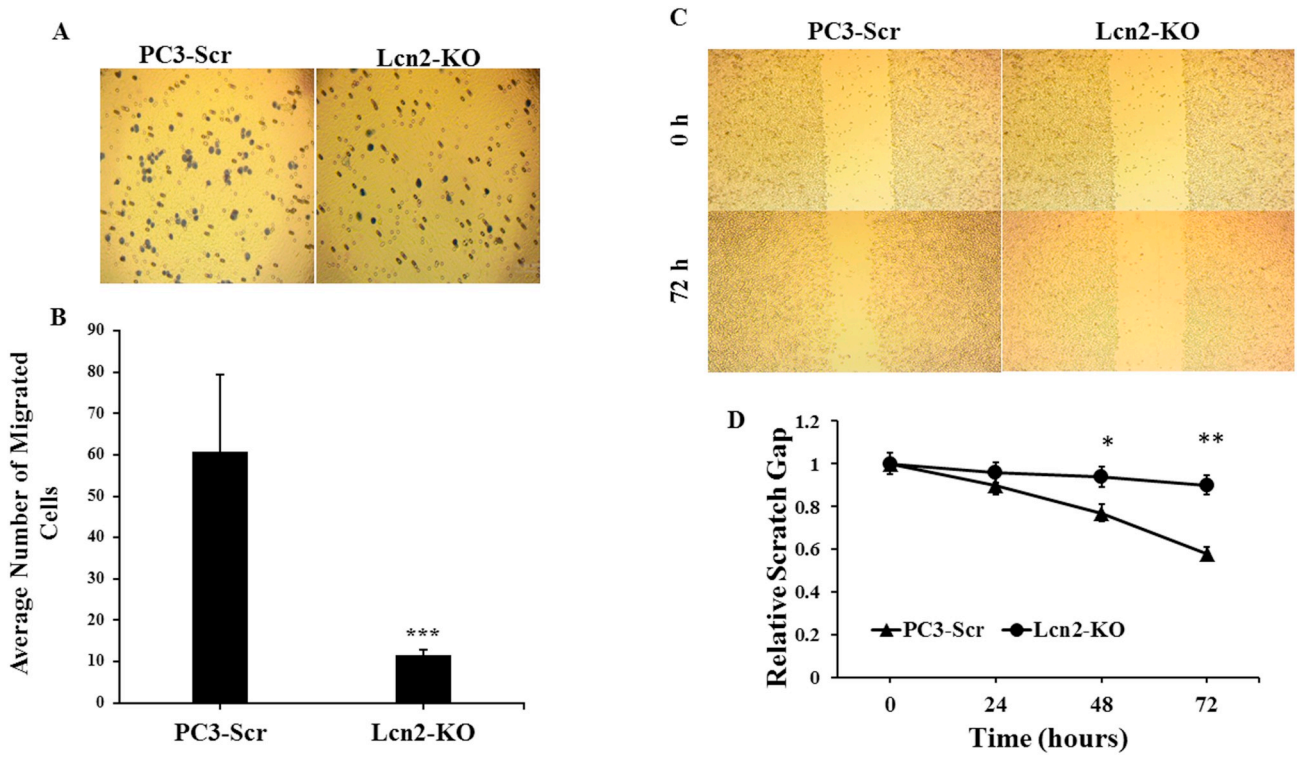

Fig. 6. Knockout of Lcn2 Remarkably Decreased PC3 Cells Migration. (A and B); for transwell assay, adequate number of PC3-Scr and Lcn2-KO cells were seeded into 8 μm transwell filters (upper chamber) in 24-well plates. After 6 h, the upper sides of the filters were gently cleaned with sterile swaps and washed by PBS. Finally the migrated cell (on the other side of the filters) fixed and stained with giemsa and the number of migrated cells was counted under a light microscope in three random fields per transwell. (C and D); for the scratch (wound) assay, equal numbers of both cells were seeded into 6-well plates. After reaching the confluency of the cells near 100%, similar scratches were drawn in each well by sterile blue pipette tips. At 0, 24, 48 and 72 h post-scratch, photos were taken from fixed regions per well. Finally, the area of each wound was analyzed and measured using ImageJ software. The results of both assays unanimously confirmed that Lcn2 has been involved in the migratory abilities of PC3 cells. (Mean ± SD, $***P < .001$, $**P < .01$, $*P < .05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

involvement of Lcn2 in promoting migration and survival against ultraviolate (UV) irradiation and cisplatin in endometrial carcinoma cells has been shown by Miyamoto et al. [23]. They also revealed that the Lcn2-induced prolonged cell survival was mediated by the phosphatidyl inositol 3-kinase (PI3K)/Akt and p53-p21 pathways. In other words, Lcn2 conferred malignant potential to the endometrial carcinoma cells.

Ding *et al* has been reported that Lcn2 has been implicated in the castration resistance by increasing androgen receptor (AR) transcriptional activity [10]. It may be of value to mention that Lcn2 has much more expression in castration-resistant prostate cancer (CRPC) – a lethal phenotype of prostate cancer– as compared with castration-sensitive prostate carcinoma. Of note, in CRPC, the tumor cells are hormone-refractory and do not respond to androgen therapy. The Ding *et al* findings strongly suggested that Lcn2 might be a novel target in CRPC therapy, however, still warrants further investigations.

5. Conclusion

Considering few exceptions including colorectal cancer in which lcn2 acts as an anti-cancer molecule, up-regulation of Lcn2 has been implicated in a variety of cancer types [8,24,34]. Lcn2 *via* several known functions including up-regulation of gelatinase, stabilization of MMP-9, anti-apoptotic, anti-oxidant, angiogenesis, and EMT, involves in cancer initiation, promotion, and also metastasis. Supporting this notion, up-regulation of Lcn2 has been considered as a valuable biomarker and prognostic factor in different cancers remarkably in prostate cancer. Moreover, Lcn2 has been shown to implicate in chemo-resistance and radio-resistance phenotypes of some types of cancer. All these unique properties of Lcn2 strongly suggest that Lcn2 might be considered as a potential, novel, and useful targeting molecule for therapeutic purposes in different cancers. In this study, Lcn2 was knocked out *via* CRISPR/Cas9 technology in a highly invasive and aggressive cancer cell line *i.e.* PC3. Our results revealed that targeting of Lcn2 expression by a robust and versatile technique *i.e.* CRISPR/Cas9 inhibited cell proliferation, increased the sensitivity of the cells to a chemotherapeutic drug, enhanced CDDP-induced apoptotic cell death, and decreased cell migration capacity of PC3 cells. However, the detailed mechanisms underlying these effects warrant further study. We suggest a potential novel modality for cancer treatment, at least for prostate cancer cells, *i.e.* targeting of lcn2 knockout *via* CRISPR/Cas9 technique and in combination with CDDP therapy. Our findings could be expanded for other cancer cells in which Lcn2 aberrantly is upregulated as well. In this account, it requires further investigations and would be the subject of future studies. It is noteworthy that in order to develop our findings as a conventional therapeutic modality, further *in vitro* studies including other prostate cancer cell lines comprising DU 145, LNCaP, 22Rv1, and NCI-H660 to expand the current findings as well as a number of preclinical and clinical trial studies are needed.

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Author contributions

SR, **AMR**, and **AE**, collected all data, samples and also accomplished all cellular and molecular tests. **MHR** controlled and managed the project, wrote the manuscript and finalized it. **YK** and **AAS** analyzed the results, read and revised the manuscript critically. All authors revised the article carefully and confirmed the edited version of the paper.

Ethical issues

There are no ethical problems for this project.

Declaration of Competing Interest

The authors declare no conflict of interest.

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