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The combination effect of Prominin1 (CD133) suppression and Oxaliplatin treatment in colorectal cancer therapy

Zahra Asadzadeh[1], Behzad Mansoori[1,2], Ali Mohammadi[3], Tohid Kazemi[4], Ahad Mokhtarzadeh[1], Dariush Shanehbandi[1], Nima Hemmat[1], Afshin Derakhshani[1,5],Oronzo Brunetti[5] Sahar Safaei[1], Marjan Aghajani[1], Souzan Najafi[1], Nicola Silvestris[5,6*], Behzad Baradaran[1,4,7]*

1. Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
2. Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
3. Department of Cancer and Inflammation Research, Institute for Molecular Medicine, University of Southern Denmark, Odense, Denmark
4. Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
5. Medical Oncology Unit-IRCCS IstitutoTumori "Giovanni Paolo II" of Bari, Bari, Italy
6. Department of Biomedical Sciences and Human Oncology DIMO-University of Bari, Bari, Italy
7. Pharmaceutical Analysis Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

*** Corresponding Authors:**

Behzad Baradaran,Ph.D, Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, 5166614731, Iran. Email: baradaranb@tbzmed.ac.ir, behzad_im@yahoo.com

Nicola Silvestris, Medical Oncology Unit-IRCCS IstitutoTumori "Giovanni Paolo II" of Bari, Bari, Italy

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Email: n.silvestris@oncologico.bari.it

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Abstract

Colorectal cancer (CRC) is considered as one of the leading types of cancer in the world. CD133, as a cancer stem cell marker,has a pivotal role in the development of drug resistance, migration, and stemness properties of CRC cells. This study designed to check the combined effect of CD133siRNA and Oxaliplatin on proliferation, migration, apoptosis, and stemness propertiesof CRC cells in HT-29 cell line.MTT assay was performed to define the combined effect of CD133siRNA and Oxaliplatin on the viability of HT-29 cells. In order to figure out the effect of this combination therapy on CD133 expression at the gene and protein level, qRT-PCR and western blot were exploited, respectively. The ability of cell migration was tested by wound healing assay as well. Also, colony formation and sphere formation wereconducted to assess the stemness properties in the combination group. Flow cytometry was conducted to investigatethe apoptosis, cell cycle, and surface expression of CD133 in different groups. Finally, the expression of migration-, and stemness-associated genes were measured by qRT-PCR.Weindicated that silencing of CD133 reduces the migration and stemness properties of colorectal cancerous cells. This suppression makes HT-29 cells more sensitive to Oxaliplatin and reduces the effective dose of thischemical drug. Therefore, the suppression of CD133 in combination with Oxaliplatin treatment might be a promising therapeutic approach in the treatment of colorectal cancer.

Key words: Colorectal cancer, CD133,siRNA,Oxaliplatin, Combination therapy

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1. Introduction

Cancer is a mainreason of death worldwide and it is estimated both the cancer incidence and mortality rates will continue to rise. Colorectal cancer (CRC) is the most prevalent malignant tumor of the digestive system and the fourth most lethal cancer worldwide[1]. Although the progression of CRC treatment has been accomplished, the 5-year overall survival rate of CRC patients is still low[2]. Therefore, the detection of novel potential biomarkers for prognosis prediction and better clarification of the precise molecular mechanisms underlying CRC malignancy might facilitate enhanced treatments for CRC patients.

It is now well-recognized that most of the tumors such as colorectal tumorscanself-renew and maintain their growth ability even after the treatment due to the presence of cancer stem cells (CSCs) [3]. Thus, drugs that target these abilities selectively, or strategies that increase their sensitivity to the usualtherapies, suggest a betterpotential for cancer treatment.One such effective strategy would be to use RNA interference (RNAi) such as small interfere RNA (siRNA) to suppress genes involved in the enhancement of these properties. siRNAs are the 20–25 base pairs of RNA molecules that actively involves in the regulation of particular gene expression throughmonitoring the gene-silencing function[4, 5].

The prominin-1 (CD133), as a CSC surface marker, is a five-transmembrane glycoprotein with 120 kDa molecular weight, and is placed in membrane protrusions. CD133 has been recognized in several cancers as a potential marker for the CSCs,including brain cancer [6], ovarian cancer[6], hepatocellular carcinoma[7], prostate cancer[8], breast cancer [9], and pancreatic cancer[10].It is demonstrated that CD133 plays a vitalrole in numerous types of cancers. CD133 has beenshown to increase migration in gallbladder carcinoma[11]. CD133upregulation contributed to the progression of hepatocellular carcinoma stimulatedthrough STAT3[12]. Also, CD133+ liver cancerous cells augmented angiogenesis, growth, and self-renewal of tumors. Furthermore, some studies have informed that CD133+ colorectal cancers may be more resistant to chemotherapy or chemoradiotherapy[3]. Moreover, two meta-analysis reports proposed that CD133 expression is notably associatedwithpoor survivaland may haveasignificant role in colorectal cancer development.

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Oxaliplatin is one of the most helpful chemotherapeutic drugs used to treat CRC in several cases. As a platinum-based anti-cancer drug, Oxaliplatin shows high activity in attachment with DNA nucleobases [13]. Regarding the interaction of Oxaliplatin and DNA in CRC cells, apoptosis pathway is excessively activated followed by DNA damage [14]. However, the cancerous cells in many CRC patients generally manifest poor sensitivity to the treatment with Oxaliplatin. CRC cells could generate some strategies to escape cell death induced by Oxaliplatin.

However, there have been few studies on the association of CD133 with the sensitivity of CRC to Oxaliplatin. Thus, the purpose of thisresearchis to examine the function of CD133 in CRC and the effectiveness of CD133 siRNA combined with Oxaliplatin therapy for CRC treatment, which could suggest a new therapeutic strategy for CRC.

2. Materials and methods

2.1. Cell lines and culture

The human CRC cell lines, HT-29, HCT-116, and SW480 were purchased from the cell bank ofPasture Institute (Tehran, Iran). Cells were grownin RPMI-1640 medium enriched with 10% fetal bovine serum (FBS; GIBCO, Carlsbad,CA) and were incubated in a 95% humidified atmosphere with 5% CO2 at 37°C.

2.2. Small interfering RNA transfection

Regarding the higher expression of CD133 in HT-29 cells compared to HCT-116 and SW480 cells, these cells were selected and seeded into six-well plates (2×105 cells per well) for transfection procedure. According to the manufacturer's protocols, jetPRIME reagent (Polyplus Co., Illkirch, France) was used to transfect 80 pmol of CD133 siRNA (Table 1), which have the better effectiveness among 20-80 pmol range of siRNA concentration and 24-72 hr range of transient transfection period, into the cells. In brief, the jetPRIME reagent and CD133 siRNAs were diluted in jetPRIME dilution buffer and placed at room temperature (RT) for 30 min. The mixtures of siRNAwere added to the wells containing cells in Opti-MEM medium. After

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incubation of plates for 5 hr at 37°C,the RPMI-1640 medium (20% FBS) was added into each well with transfected cells.

2.3. Evaluation of gene expressions by quantitative RT-PCR

RNA was isolated from the cells via RiboEX reagent (GeneAll Biotechnology, South Korea, Seoul) according to the manufacturer's instruction. Next, the total RNA concentration was considered by Nanodrop (Thermo Fisher Scientific, Lenexa, KS). Then, 1 µg of total RNA was used to synthesize complementary DNA (Biofact, South Korea)via thermal cycler system (Bio-Rad, Hercules, CA). Before the experiment, all pair primer sequences were blasted by means of the primer-blast software of the NCBI website(http://www.nchi.nlm.nih.gov). SYBR green master mix and specific primers of CD133, MMP-9, VEGF, E-cadherin, β-catenin, Sox2, Oct4, IGF-2, and MDR1were used to perform real-time PCR (Table 2). Also, 18s rRNA expression was considered as a reference gene. The mRNA expression was evaluated by STEP ONE PLUS qRTPCR system (Applied Biosystems, Foster City, USA) and interpretedthrough the $2^{-\Delta\Delta Ct}$ method.

2.4. Proliferation assay

MTT assay was performed to examine the viability of HT-29 cells after using CD133 siRNA and Oxaliplatin, individually and in combination. In brief, 15×10^3 HT-29 cells were plated into 96-well plate. Then,optimal concentrations of the CD133 siRNA were used to transfect cells, individually and in a combination with Oxaliplatin.After 48hr, 50 µlof MTT (2 mg/ml) were added to the well for 2- 4 hr. After removal of the medium, 150 µlDMSO was added in each well and shaken for 10 min. The optical density (OD) of each well was evaluated at 570 nm with an ELISA reader (Sunrise RC, Tecan, Switzerland).

2.5. Evaluation of protein levels by western blotting

HT-29 cells were seeded in to six-well plates at a density of 2×10^5 cells per well. Four groupsincluding control, CD133 siRNA, Oxaliplatin, and combined CD133/Oxaliplatin were considered. In order to extract the total protein, RIPA lysis buffer (Santa Cruz

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Biotechnology,Santa Cruz, CA) wasconsidered. The cellpellet was resuspended in lysis buffer, containing the proteaseinhibitor, phosphatase inhibitor, and PSMF. Cell lysates were centrifuged at 13,000 rpm for 15 min at 4°C. Subsequently, protein samples were subjected to 12.5% SDS-PAGE electrophoresis, and transferred to polyvinyl difluoride (PVDF; Roche Diagnostics) membranes.Then, these membranes wereblocked with Tween 20 (0.5%) in PBS for 2 hr in RT and then incubated with an anti-CD133 monoclonal antibody (Abnova, Taipei, Taiwan) (1: 1,000) and β-actin (Abcam) as the reference (1:5,000); (Santa Cruz Biotechnology, Santacruz, Ca). Then, the membranes were incubated for 1 hr at RT with rabbit anti-goatsecondary antibody for CD133 and rabbit anti-mouse antibody for β-actin conjugated with horseradish peroxidase (1:5,000; diluted in PBS). The specific bands wereidentified by the Roche Diagnostics electrochemiluminescence (ECL) kit and western blot imaging system (Sabz Co., Iran). Ultimately, Image J software (National Institutes of Health, Bethesda, MD) was employed for evaluation.

2.6. Flowcytometry analysis of surface expression

To evaluate the cell surface expression, 2×10^5HT-29 cells were seeded in each well of six-well plates and were grown in a humidified incubator for 24 hr. Next, four groups were described: control, CD133 siRNA, Oxaliplatin, and CD133 siRNA/Oxaliplatin. After 24 h of CD133siRNA transfection, cells were treated with Oxaliplatin. After 24 hr incubation at 37°C, cells were centrifuged and washed twice in FACs buffer. Next,the primary antibody (100 µl) was added and cells were incubated for 30 min at RT in the dark. Then, the FITC conjugated-secondary antibody (1:1000) was added to the samples and they incubated in the dark condition for 20 min.

2.7. Flowcytometry analysis of apoptosis

Apoptosis was evaluated by annexin V/propidium iodide (PI) assay. First, 2×10^5HT-29 cells were seeded in each well of a six-well plate.After 24 hr incubation,the cells were separated to four different groups, including control, CD133 siRNA, Oxaliplatin, and combined CD133/Oxaliplatin groups.After 24 hrof CD133 transfection, the cells were treated with Oxaliplatin and incubated for 24 hr.Then, the cells were stained with annexin V and PI according

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to the manufacturer's protocols(EXBIO, Vestec, Czech Republic). For annexin V staining, 200 µl of binding buffer, annexin V (5 µl), and propidium iodide (5 µl) were added to the different groups. Next, they were incubated for 20 min at RTin the dark. Then, the groups were evaluated by flow cytometery (MiltenyBiotec™ FACS Quant 10; MiltenyBiotec, Germany). The apoptosis rate was measured by FlowJo software (Tree Star, San Carlos, CA).

2.8. Flowcytometry analysis of cell cycle arrest

First, 2 × 10^5 HT-29 cells were seeded in to each well of 6-well platesand were grown in a humidified incubator for 24 hr. Then, four groups were described: control, CD133 siRNA, Oxaliplatin, and combined CD133/Oxaliplatin.After CD133-siRNA transfection and Oxaliplatin treatment, the cells were prepared for analysis.Theywere washed with PBS, and 70% ethanol was used to fix them overnight (−20 °C). Next, after washing of the cells with PBS, they were resuspended in PBS containing RNase A (200 µg/ml), incubated at 37 °C for 30 min, and stained with DAPI (50 µg/ml) for analysis. The distribution of cells in each phase of cycle was evaluated using a Flowcytometry instrument (MiltenyBiotecMACS Quant 10) and the data were analyzedby the FlowJo FACS analysis software.

2.9. Scratch-wound migration assay

Migration of HT-29 cells was analyzed by the wound-healing assay. The HT-29 cells were seeded in 24-well plates (3 ×10^5 cells/well) and grown to 90% confluence. Then,four groups, including control, CD133 siRNA, Oxaliplatin, and combined CD133 siRNA/Oxaliplatin were considered. The cell monolayers were scratched using a 2 mm-wide tip to form a line-shaped wound.Next, the cells were transfected and treated with CD133siRNA and Oxaliplatin, respectively. The cells were permitted to migrate, and images were collected at different time (0, 24, and 48 h) by an inverted microscope.

2.10. Colony formation assay

Colony formation ability was evaluated by culturing the cells in 3D cell culture mediumand groups,including control, CD133 siRNA, Oxaliplatin, and combined CD133/Oxaliplatin was

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investigated in HT-29 cells. So, 5×10^3 of the cells were seeded into six-well plates. The cells were transfected with siRNA and treated with Oxaliplatin.After 12 days,staining dye (including crystal violet, formaldehyde, and methanol) were used to stain the colonies for 30 min. Ultimately, the number of colonies for each groupwere counted using ImageJ software (NIH, MD).

2.11. Spheroid assay

Sphere formation ability of different groups, including control, CD133 siRNA, Oxaliplatin, and combined CD133/Oxaliplatin was assessed in HT-29 cells. To this aim, these cells were seeded into six-well plates at a density of 2×10^5 cells/ well. After CD133siRNA transfection and Oxaliplatin treatment of cells, they were incubated for 24 hr at 37°C.Then, the cells were trypsinized and transferred to 96-well plates. In a 96-well plate, a well was considered for each group and 2x concentrationof RPMI-1640 medium (Sigma, USA) containing matrigel 10%were added in wells. Next, 5×10^3 cells were seeded from each group of treated cells in each well of the 96-well plate and incubated at 37 ° C. The image of spheres indifferent groups were captured by an OPTIKA (Italy) microscope system for 10 days.

2.12. Statistical analysis

All data analyses were performed on GraphPad Prism software version 7.0 (GraphPad Prism; San Diego, CA). Measurement data were expressed as mean ± standard deviation. Unpaired student's t-test and ANOVA one-way analysis of variance was used for comparing the groups with parametric data. $P < 0.05$ was depicted as statistical significance.

3. Results:

3.1.CD133 is overexpressed in colorectal cancer cell line, HT-29

CD133 expression was investigated in various CRC cell lines HT-29, HCT-116, and SW-480. qRT-PCR results revealed that the expression of CD133 in the HT-29 cell line is significantly higher than HCT-116 and SW-480 cell lines (Fig.1A).

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3.2. CD133 siRNA reduced the expression of CD133 in HT-29 cells

To evaluate the CD133 activity in HT-29 cells, we performed a siRNA strategy. HT-29 cells were transfected with various doses (20-80 pmol) of CD133-siRNA. After transfection of HT-29 cells with 80 pmol dose of CD133 siRNA, the cells indicateda significant low expression of CD133 (Fig. 1B).The dose of 80 pmol was selected for all next experiments to knockdown HT-29 cells. Next, the transfection of HT-29 cells was done by CD133siRNA for 24, 48, and 72 h. Finally,48 h were considered as the optimum knockdown time (Fig. 1C). The qRT-PCR analysis demonstratedthat transfected cells have lower expression of CD133 mRNA compared to control cells.

3.3.CD133 knockdown has no significant effect on cell viability

MTT assay was conducted to investigatethe viability and proliferation of HT-29 cells after CD133siRNA transfection.This indicatd that, transfection with 20–80 pmol of CD133siRNA after 48 h incubationhas no significant effect on cell viability or proliferation compared to the control group (Fig. 2A).These results propose that CD133 is not associatedwith cell viability of HT-29 cells.

3.4. Combination of CD133siRNA and Oxaliplatin decreased cell viability in HT-29 cells

To consider the effect of CD133siRNA and Oxaliplatinon proliferation of HT-29 cells, MTT assay was conductedto find the IC50 value of Oxaliplatin (IC50 =32.85). The results revealed that transfection of CD133siRNA lessens the IC50 value of Oxaliplatin (IC50 =19.75). So, it isrevealed that the combination of CD133siRNA and Oxaliplatinmakes a notable alteration in proliferation of cells (Fig. 2B). These resultsexposed that the combined use of CD133siRNA and Oxaliplatin simultaneously reduces the proliferation rate of HT-29 cells.

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3.5. The combined use of CD133siRNA and Oxaliplatin decreased the levels of CD133 mRNA and protein in HT-29 cells

We next used CD133 siRNA in combination with Oxaliplatin in the HT-29 cell line. First, we used CD133 siRNA and Oxaliplatin separately. qRT-PCR and western blot analysis detected that the individual CD133 siRNA and Oxaliplatin results in reduced CD133 mRNA and protein expression levels. While, the combined CD133siRNA and Oxaliplatinsuppressed the CD133 mRNA and protein level more than individual using (Fig. 3A,B, C).

3.6. Combination of CD133-siRNA and Oxaliplatin decreased surface expression of CD133 in HT-29 cells

Combined usage of CD133 siRNA and Oxaliplatin led to a decrease in CD133 surface expression. The flowcytometric analysis revealed a decreased percentage of $CD133^{+}$ cells in individual groups of CD133 siRNA and Oxaliplatin, 15.3 and 32.4 respectively. While the combined group exhibited a lower percentage of $CD133^{+}$ cells 2.41, compared with the other groups (Fig. 3D, E).

3.7.Knockdown of CD133 in combination with Oxaliplatin could sensitize the apoptosis ofCRC cells

To investigate if single CD133 siRNA or combination of CD133 siRNA/Oxaliplatin increases apoptosis, annexin V/PI assay was performed. We indicated that dual usage of CD133 siRNA and Oxaliplatin induces apoptosis more than individual usage of CD133 siRNA and Oxaliplatin. The apoptosis rate for CD133 siRNA and Oxaliplatinwere2.92%and 3.85% in HT-29 cells, respectively. Interestingly, the rate of apoptosis following CD133 silencing in combination with Oxaliplatin was higher: 5.41%(Figure 4A,B). Thus, we detected cooperative increases in apoptosis after combined usage of CD133 siRNA and Oxaliplatin.These datapresented that the silencing of CD133 could decrease the Oxaliplatin resistance ofHT-29 cells.

3.8.Combination of CD133 siRNA/OxaliplatinarrestedHT-29cells in G1 phase of cell-cycle

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To further determine whethercombination ofCD133 siRNA and Oxaliplatinaffects cell proliferation, we examined cell-cycle distribution of HT-29 cells after CD133siRNAtransfection and Oxaliplatintreatment. Flow cytometry data indicated thatCD133-siRNA augmented the population of G1 phase cells from 76.2% to 81.8%. Also, treatment with Oxaliplatin resulted in a rise in the population of G1 phase cells by 76.2% to 83.0% compared to controls. Interestingly, CD133 siRNA combined with Oxaliplatin augmented the population of cells that were in G1 phase from 76.2% to 91.1%. It is indicated that combination is able to enhance the percentage of HT-29 cells in the G1 phase.Actually, CD133siRNAandOxaliplatindual usage could supportthe arrest of cellcycle in the G1 phase (Figure 4C,D).

3.9.Silencing of CD133 expression in combination with Oxaliplatin decreasedstemness features in HT-29 cells

To recognize if individual and/or combined usage of CD133 siRNA and Oxaliplatin could dampen stemness properties, we performed colony formation assay. In comparison to the control cells, the colony numbers were decreasedafterCD133 siRNA and Oxaliplatin single usage. While, the colony numbers were remarkably low in the combination group(Fig. 5A, B). Also, the result of the sphere formation assay demonstrated that using combined CD133 siRNA and Oxaliplatin leads to forming spheroids with smaller diameter compared with the individual usage of CD133 siRNA and Oxaliplatin (Fig. 5C).To support the reduction of stemness features following CD133 siRNA transfection at the molecular level, we examined the expressionof stemness related genes. qRT-PCRassays presented that Oct4, Sox2, MDR1, and IGF2 mRNA expression levelswere meaningfullylessened in CD133 siRNA/Oxaliplatin combination group compared with the individual CD133 siRNA and Oxaliplatin groups (Fig.5D, E, F, G). Therefore, both phenotypic andmolecular genetic dataillustrates that silencing CD133 expression in combination withOxaliplatin decreases the stemness features of colorectal cancercells.

3.10.CD133 siRNA and Oxaliplatinwork together to inhibitemigration and EMT-related genes expression in HT-29 cells

The result of the wound healing assay indicated that the combination of CD133 siRNA and Oxaliplatin reducesmigration of HT-29 cells.Single usage of CD133 siRNA

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andOxaliplatindecreased the migration of these cells. However, compared to the control cells, double usage ofCD133 siRNA and Oxaliplatin, 48 h after transfectionsignificantly reduced migration rate of HT-29 cells (Fig.6A, B). To assess if this combinationalters the expression of genes associated with EMT, we investigated the expression of VEGF, MMP-9, E-cadherin, and β-cateninby qRT-PCR. This assay showed that the relative expression levels of VEGF, MMP9, and β-catenin geneswere lessened inindividual usage and were remarkably lowaftercombinationusage. While E-cadherin indicated increased expression in the combination group (Fig.6C, D, E, F). Thus, individual usage of CD133 siRNA and Oxaliplatin reduces cell migration and EMT-related genes expression and dualusage enhances these effects.

4. Discussion:

Most ofthe cancerous cells have the ability to self-renew, differentiate into established progenies, and induce and preserve tumor development even after the treatment[15]. CD133 is one of the most well-known markers for the evaluation of CSCs[16].It isrevealed that CD133 promotes the growth and invasion of cancerous cells. According to studies, knockdown of CD133 lessens the development of hepatocellular carcinoma cells [17]. CD133 facilitates cell proliferation in colon cancer as well[18]. Moreover, CD133 downregulation stops the self-renewal and tumorigenesis of glioma cells [19]. These results indicate that CD133 might be a molecular target for successful cancer treatment. Itis indicated that the combination of gene therapy and anti-cancer drugs can be a successful treatment strategy. In this investigation, we analyzed the combined effect of CD133 siRNA and Oxaliplatin in HT-29 cell line of colorectal cancer.

It is reported that CD133 reveals a notable therapeutic impact in increasing the sensitivity of chemotherapy in many cancers.Several studies have been conducted to evaluate the effect of CD133 silencing on drug sensitivity. Zhang et al.demonstrated that suppressed CD133 improves the efficiency of cisplatin in laryngeal carcinoma treatment[20]. Also, CD133 downregulation reduced the drug resistance of oral squamous cell carcinoma (OSCC) to cisplatin. Synergistic effects of CD133 suppressing and cisplatin chemotherapy weakened tumor-initiating cells characteristic inOSCC as well[21]. A recent study showed that inhibition of CD133 increased the sensitivity of Cis-KATO-III cells to cisplatin through PI3K/AKT/mTOR signaling network[22] Le et al. confirmed thatCD133 overexpression leads to the resistance of malignant cells to the 5-FU- or cisplatin-induced cell death in head and neck squamous cell carcinoma (HNSCC) [23].In

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the present study, the results of cytotoxicityassay exhibit that the effective dose of Oxaliplatin is decreased in combination with CD133 siRNA. In other words, it can be concluded that CD133 siRNA enhances the sensitivity of the HT-29 cells to Oxaliplatin. Also, it increases the stimulation of cell death by inducing a synergistic effect and increasing the drug effect in the HT-29 cancer cell line.

The results of Annexin-V/PI staining confirmed the MTT results and demonstrated that CD133 siRNA did not play a substantial role in thestimulation of apoptosis. However, the rate of apoptosis in the combination of CD133 siRNA and Oxaliplatin was meaningfullyaugmented. It can be stated that CD133 siRNA induces apoptosis in CRCsby increasing the sensitivity of these cells to the Oxaliplatin.Someresearchesrecognized that CD133 canstopapoptosis and stimulatedrug resistance throughtriggeringthe PI3K/AKT signaling network in gastric and hepatocellular cancer cells[22]. Moreover, a research presented that CD133 suppression had no importantimpact on cell proliferation or apoptosis. while, CD133 knockdown leads to thehigh susceptibility to staurosporine-induced apoptosis[24].Using flow cytometry for cell cycle analyses, we indicated that the cells withinthe G0-G1phase are increased in CD133-silenced cells after treatment with Oxaliplatin.Lan et al.revealed that the $CD133^{+}$Liver cancer cells were generally distributed in the G0/G1. Following the CD133 downregulation by RNAi, cells in the G0/G1 phases of the cell cycle were reduced, which exposed that CD133 inhibitioninducesliver cancerous cells to differentiate [25].

Metastasis is a mechanism by which tumor cells separate from the preliminary tumorplaces into the stroma and enter blood vessels. This process is accompanied by the development of a secondary tumor at a distant place. To examine the mechanism of CD133 by which the cell migration is adjusted, Liu et al. studied the $CD133^{+}$ colorectal cancer cell line SW620 and $CD133^{-}$ cell line HEK293T. Suppression of CD133 lessened cell migration and FAK phosphorylation in SW620 cells. In HEK293T cells, ectopic expression of CD133 increased cell migration and the phosphorylation level of FAK at the Tyr925 site. Additionally, they reported that CD133 promotesmigration via its interaction with Src, stimulating the phosphorylation of FAK[26].In another study, Sohn et al.indicated that CD133 has animportant role in the increasing of bone metastasis by enhancing EMTin a prostate cancer cell line, LNCaP.Ectopic overexpression of CD133 in LnCaP cells changed EMT characteristics, including decreased E-cadherin and augmented vimentin expression, wound gap distance, and cell

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migration[27].Also, Nomura et al.presented that the augmented metastatic ability of $CD133^{+}$ cells is facilitated by the stimulation of the NF-κB pathway and NF-κBstimulationaugmented invasion and triggered EMT[28]. Similarly, the downregulation of CD133 suppresses the migration and invasion of gallbladder carcinoma through decreasing Akt phosphorylation [2]. In the case of Oxaliplatin, Li et al.results presented that Oxaliplatininhibits hepatocellular carcinoma cell proliferation and migration ability by upregulating GAS7C and inducing the N-WASP/FAK/F-actin pathway [29]. One of the significant results of this research is that the inhibition of CD133 represses migration in colorectal cancer. The wound healing assay results indicated that the individual use of either CD133 siRNA or Oxaliplatin lessens migration. However, combined use of CD133 siRNA and Oxaliplatin was relatedto a significant reduction in migration compared to the control group, indicating the synergistic effect of CD133 siRNA and Oxaliplatin.Thus, regarding the results of the present study and the previous researches, it could be proposed that the combined effect of CD133 siRNA and Oxaliplatin decreases migration in colorectal cancer cells.

According to currentstudies, CD133 also demonstrated a fundamental role in the stimulation of stemnessproperties in various cancers. It is indicated that CD133 overexpression supports stemness characteristics and tumorigenicity of HNSCC. While, CD133 inhibition stimulatesSrc activation and decreases stemness properties and tumorigenicity of HNSCC both in vitro and in vivo [30]. Furthermore, it is demonstrated that theCD133 overexpression isrelated to the stemness of oral squamous cell carcinoma (OSCC).Genetic modulation of CD133 changed the expression of some stemness related genes, such as OCT4, SOX2, and NANOG, leading toaugmented colony-forming characteristics. These resultsproposed that different states of CD133expression affects stemnessproperties [31]. In a study conducted byLan et al.,it isexhibited that $CD133^{+}$ liver cancerous cells have greater colonyforming capacity than the $CD133^{-}$ cells. CD133-expressing cells developedfurther and larger colonies than $CD133^{-}$cells,suggesting that CD133 functions as a vitalfactor in sustaining the liver cancer cells performances [25].According to the results of colony formation assay, the individual use of CD133 siRNA or Oxaliplatin decreases the colony numbers. However, dual usage of CD133 siRNA and Oxaliplatin meaningfullydiminished the number of colonies compared to the control group, supporting the synergistic effect of CD133 siRNA and Oxaliplatin.The result of the sphere formation assay also confirmed the result of colony formation. Individual use of siRNA

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or Oxaliplatin decreased the size of spheroids, which was more significant in the combination group.

In conclusion, this study revealed that the silencing of CD133, as a CSC marker,promotes the sensitivity of colorectal cancer cells to Oxaliplatin and subsequently stimulates cell death, suppresses proliferation, and migration by stimulatingthe synergistic effect. The knocking down of CD133 could act as a possiblestrategy to sensitize the cancer cells to Oxaliplatin in the treatment of colorectal cancer.

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Conflict of interest

The authors declared that there is no conflict of interest related to this study.

Author Contributions:

Z.A., the first author of the manuscript, performed the experiments and wrote the primary version of manuscript.B.M., A.M., D.S., A.M., and T.K.,helped in performing the experiments., N.H., A.D., O.B., improved the quality of the paper. S.S., M.A. andS.N.: contributed to English editing of the manuscript and also helped with data categorization. B.B. and N.S.: the corresponding authors of the manuscript, contributed to the writing of the main text of the manuscript and also supervised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Table legends:

Table 1. Three different CD133 siRNA sequences.

Table 2. Primer sequences.

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Tables:

Table1.

siRNA duplexes	Sense	Antisense
CD133 siRNA (1)	5′-UUG UCA UAA UCA AUU UUG GTT-3′	5′-AAC AGU AUU AGU UAA AAC CTT -3′
CD133 siRNA (2)	5′-UGA AGU UCU GAG CAA AAU CTT-3′	5′-TTG AUU UUG CUC AGA ACU UGA-3′
CD133 siRNA (3)	5′-AGA AAG UCC UAU AAU ACU CTT-3′	5′-TTG AGU AUU AUA GGA CUU UCU-3′

Table2.

Target	Forward primer	Reverse primer
CD133	5′-AAT GGA TTC GGA GGA CGT GTAC-3′	5′-AGC ACT ACC CAG AGA CCA ATG-3′
MMP-9	5′-TTG ACA GCG ACA AGA AGT GG-3′	5′-GCC ATT CAC GTC GTC CTT AT-3′
VEGF	5′-TGCAGATTATGCGGATCAAACC-3′	5′-TGCATTCACATTTGTTGTGCTGTAG-3′
E-cadherin	5′-TGC CCA GAA AAT GAA AAA GG-3′	5′-GTG TAT GTG GCA ATG CGT TC-3′
β-catenin	5′-GCGTGGACAATGGCTACTCAAG-3′	5′-AGGTATCCACATCCTCTTCCTCAG-3′
Sox2	5′-ACATGTGAGGGCCGGACAGC-3′	5′-TTGCGTGAGTGTGGATGGGATTGG-3′
Oct4	5′-GGGCTCTTTGTCCACTTTGT-3′	5′-GGCATGCATACACACAAACAC-3′
IGF-2	5′-AACGAGTGGAGGGGATGAGG-3′	5′-AGGAGAGGGACAAAGCTGAGG-3′
MDR1	5′-TTCCGCTTCTTCGTCTGCTT-3′	5′-TCTTGCCATCTTCCGACCAC-3′
18s	5′-GAT CAG ATA CCG TCG TAG TTCC -3′	5′-CTG TCA ATC CTG TCC GTG TC -3′

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Figure legends:

Fig. 1:A. The expression of CD133 in HT-29, HCT-116, and SW-480 cell lines. The expression of CD133 in HT-29 cell line is higher than HCT-116 and SW-480 cell lines. **B, C. The effect of siRNA on the expression of CD133**. The CD133 expression level in HT-29 cells, which affected by different doses of siRNA, were decreased the most at the dose of 80 pmol. CD133 siRNA showed the highest rate of suppression at 48 hours after transfection (**p< 0.01, ***p< .001, ****p< 0.0001).

Fig. 2:The effect of CD133 siRNA on HT-29 cell survival.A. The usage of CD133 siRNA at different doses did not have a considerable effect on the survival rate of transfected cells. **B.**Usage of CD133-siRNA and Oxaliplatin in combination caused a significant difference in IC50 value.

Fig. 3:The effect of CD133 siRNA and Oxaliplatin combination on the CD133 expression at mRNA and protein level as well as the surface expression.A.CD133 siRNA in combination with Oxaliplatin reduced CD133 mRNA expression. **B.**Western blot analysis showed decreased CD133expressionat protein level in the combination group. **C, D.** combined CD133 siRNA and Oxaliplatin significantly lessened the surface expression level of CD133(*p< 0.05, **p< .01, ***p< .001, ****p< 0.0001).

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Fig. 4:**The effect of CD133 siRNA in combination with Oxaliplatin on apoptosis and cell cycle.A, B.** CD133 knockdown in combination with Oxaliplatin sensitized HT-29 cells to apoptosis.**C, D.** Combined usage of CD133 siRNA and Oxaliplatin caused cell-cycle arrest in the G1 phase(*p< 0.05, **p< .01, ***p< 0.001).

Fig. 5: The effect of CD133 siRNA in combination with Oxaliplatin on stemness features.A, B, C. The colony numbers and the diameter of the spheroids were significantly decreased in the combination group. **D, E, F, G.**Furthermore, the expression of stemness related genes (Oct4, Sox2, MDR1, and IGF2)were decreased in dual usage group(*p< 0.05, **p< .01, ***p< 0.001, ****p< 0.0001).

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Fig. 6: The effect of CD133 siRNA in combination with Oxaliplatin on metastasis.A, B. Combination of CD133 siRNA and Oxaliplatin, 48 h after transfection meaningfully lessened the migration rate of HT-29 cells compared to the control cells. **C, D, E, F.** Also, the expression of metastasis-related genes, including VEGF, β-catenin, and MMP9 were decreased and E-cadherin expression increased in combination group(*p< 0.05, **p< .01, ***p< 0.001, ****p< 0.0001).

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Fig 1.

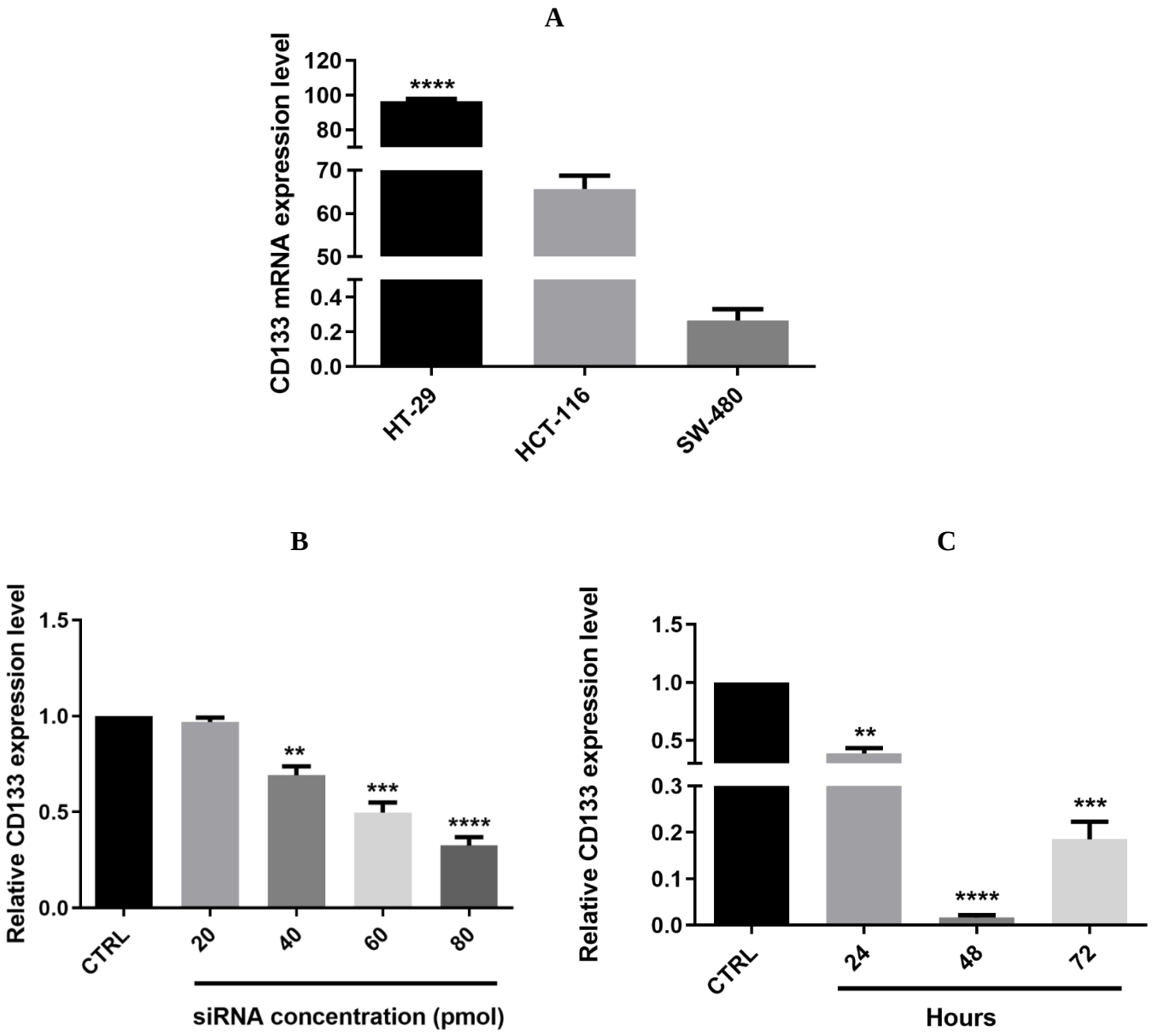

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Fig2.

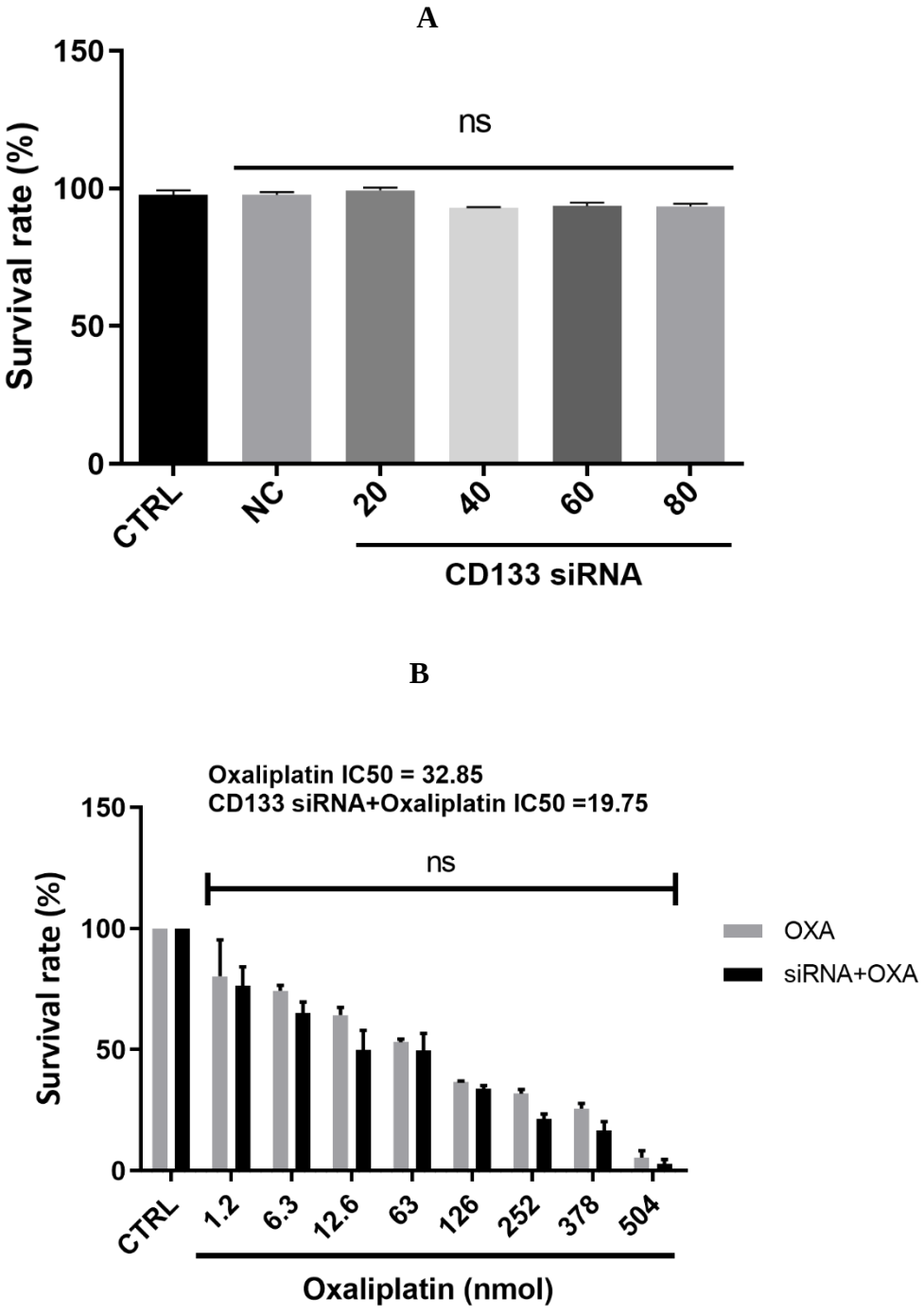

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Fig.3.

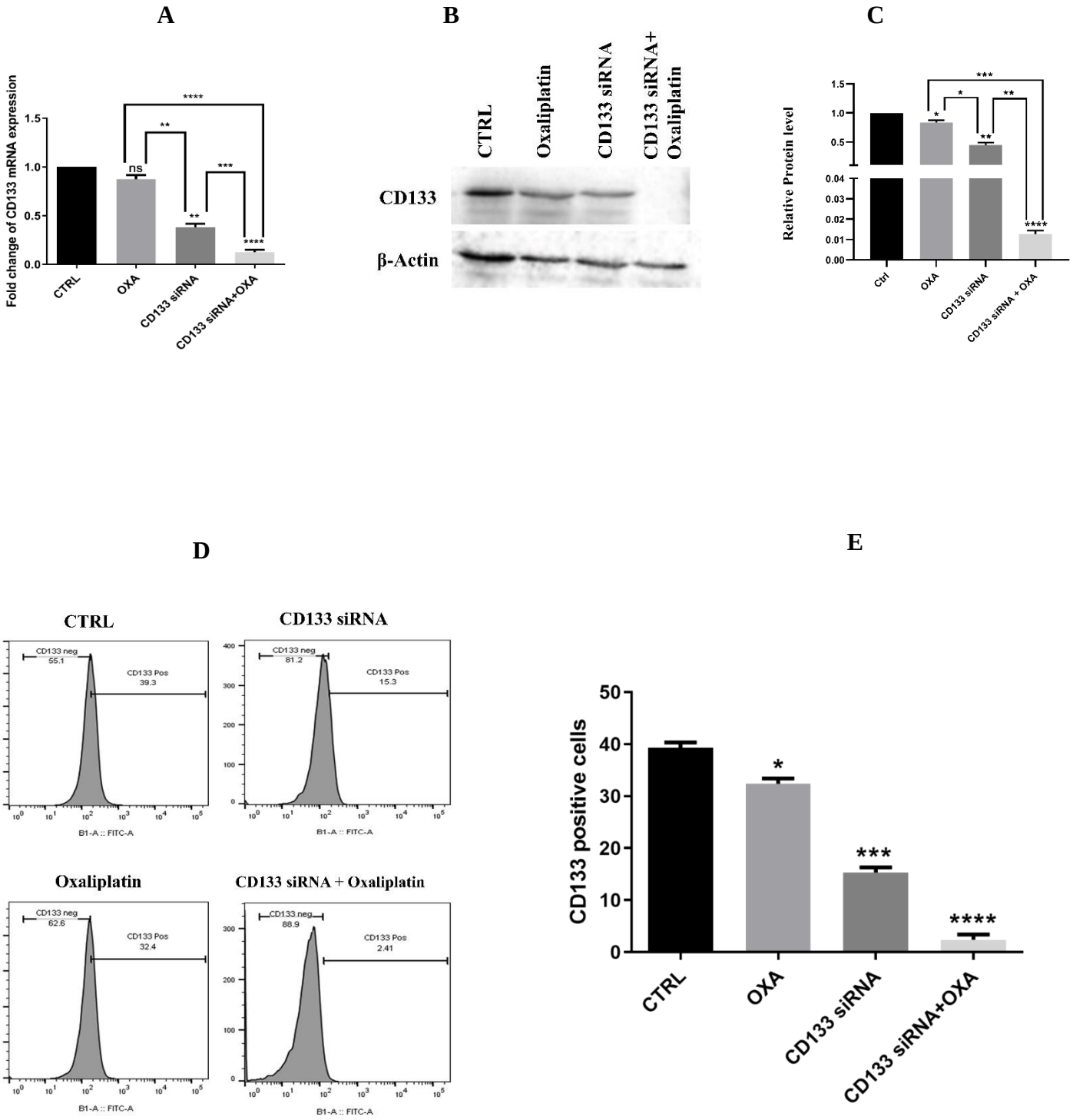

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Fig4.

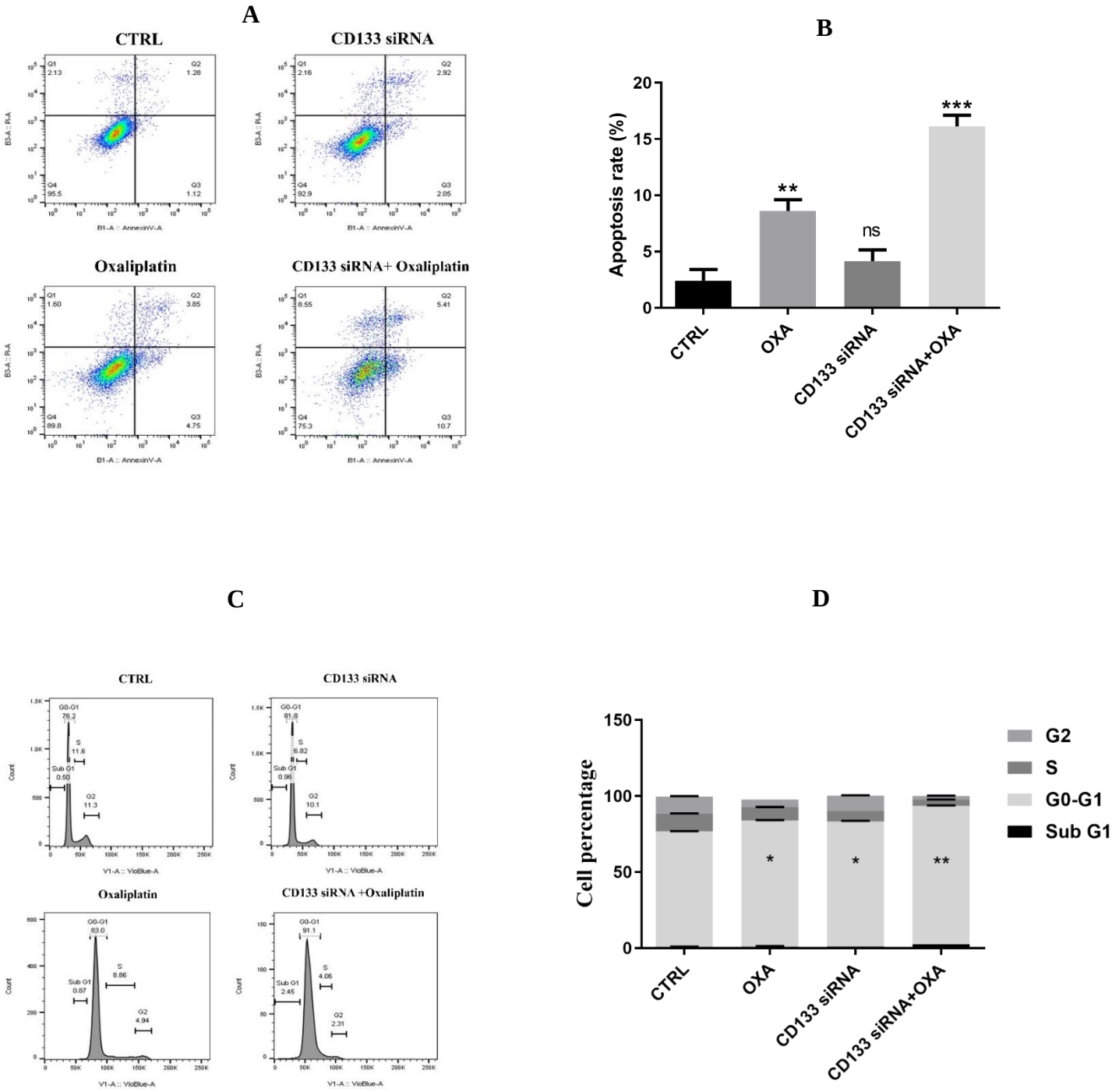

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Fig5.

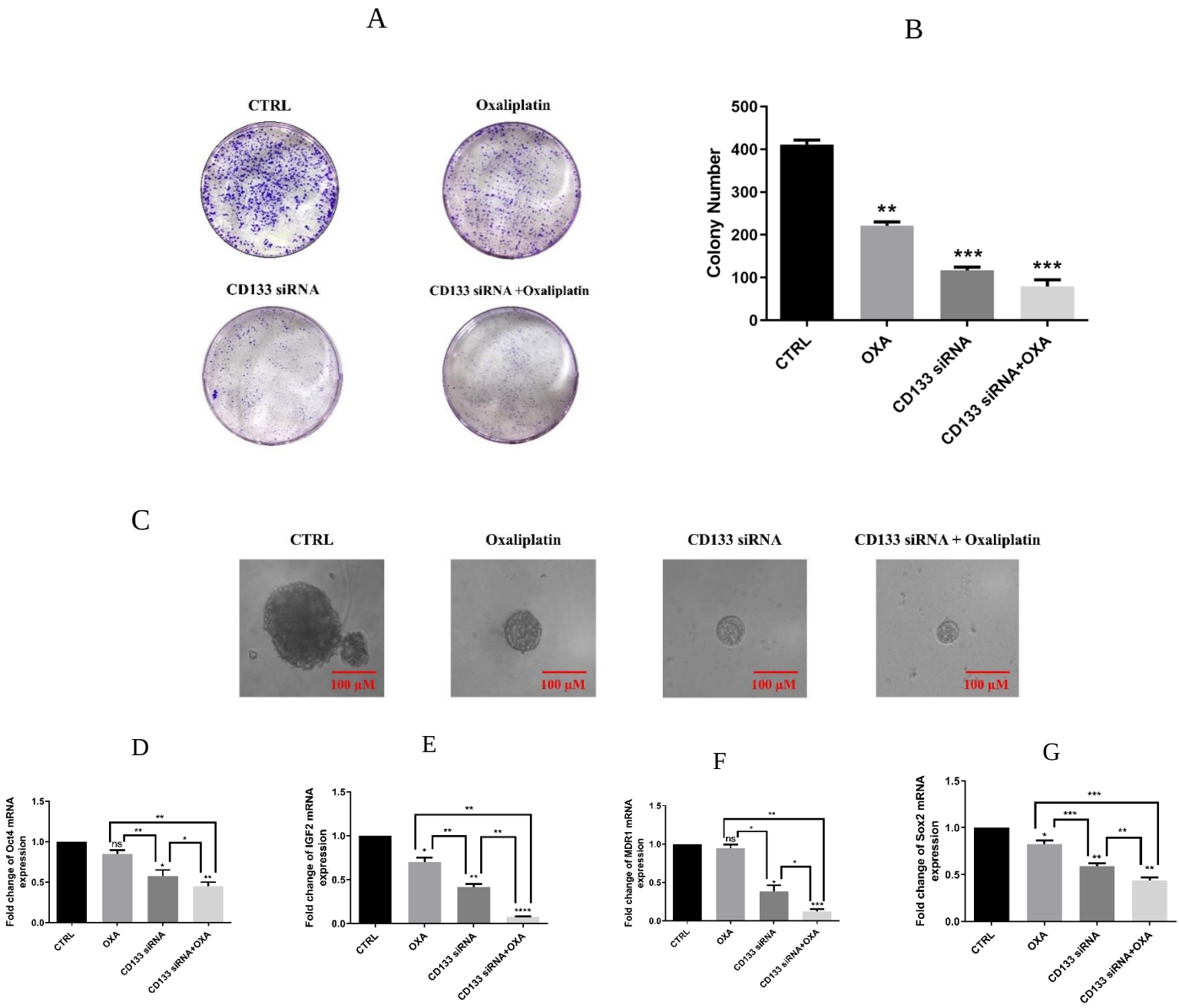

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Fig.6.