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Lidocaine sensitizes the cytotoxicity of 5-fluorouacil in melanoma cells *via* upregulation of microRNA-493

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Lidocaine is a well-documented local anesthetic that has been reported to sensitize the cytotoxicity of cisplatin in cancer cells. However, little information is available concerning whether lidocaine sensitizes the cytotoxicity of 5-fluorouracil (5-FU) in melanoma cells. The study was aimed to explore the effects and mechanisms of lidocaine on the sensitivity to 5-FU in the melanoma cell line SK-MEL-2. Cell viability and apoptosis were analyzed after administration of different concentrations of lidocaine, 5-FU, or the combinations. Expression of microRNA (miR)-493 was assessed following lidocaine administration. The target genes of miR-493 were verified by luciferase reporter assay, PCR, and Western blot. The effects of abnormal expression of miR-493 and/or SRY-Box 4 (SOX4) on cell viability, apoptosis, and key proteins in phosphatidylinositol-3-kinase (PI3K)/AKT and the Smad pathways were detected. The effects of (0-100 uM) lidocaine on cell viability and apoptosis was not obvious; however, lidocaine could significantly increase the cell viability and inhibit apoptosis in 5-FU-treated cells. In addition, lidocaine induced upregulation of miR-493 in a dose-dependent manner, and we confirmed that the effects of miR-493 on the sensitivity were by upregulating miR-493. Moreover, we verified that Sox4 was a target of miR-493, and Sox4 overexpression decreased the sensitivity to 5-FU. Besides, Sox4 overexpression increased the levels of p-PI3K, p-AKT, p-Smad2 and p-Smad3, and Sox4 suppression showed contrary results. Our results suggest that lidocaine sensitizes the cytotoxicity of 5-FU in melanoma cells via upregulation of miR-493, which might be involved in SOX4-mediated PI3K/AKT and Smad pathways.

1. Introduction

Cutaneous melanoma is one of the most aggressive forms of cancer which arises from the melanocytes (Bataille et al. 2004). Melanoma most commonly occurs in people aged between 30 and 60 years. White people are at more risk of developing melanoma. The other common risk factor includes sun exposure (Bataille et al. 2004; Hodis et al. 2012). Common treatment options include surgical resection of the lesions, immunotherapy with high-dose interferon therapy, chemotherapy (with drugs like dacarbazine, temozolomide, vemurafenib, and dabrafenib) and radiation therapy (Bataille et al. 2004; Hodis et al. 2012; Maverakis et al. 2015; Russo et al. 2014; Thomas et al. 2006). 5-Fluorouracil (5-Fu) is a known anticancer drug which has shown efficacy against different types of skin cancer melanocytic dysplasia and lentigo maligna melanoma (Paolino et al. 2008).

Lidocaine, a local anesthetic agent, is used for the management of cancer-related pain (Uzaraga et al. 2012); however, studies have described toxic effects of lidocaine on different types of cells including melanoma cells and breast cancer cells (Chlebowski et al. 1982; Kang et al. 2016; Lirk et al. 2012). In SHG cell line (human melanoma cell line which is derived from malignant ascites), lidocaine and procaine have shown similar anti-tumor effects as observed with doxorubicin, and even a combination of non-inhibitory doses of procaine with doxorubicin have shown marked suppression of growth of SHG cells (Chlebowski et al. 1982).

In the present study, we explored the role of combination therapy of lidocaine with 5-FU in melanoma cells.

2. Investigations and results

2.1. Effects of different concentrations of lidocaine on melanoma cell viability and apoptosis

Cell viability of the SK-MEL-2 human melanoma cells was assessed following treatment with different concentrations of lidocaine (10 μM, 100 μM, and 1000 μM). It was found that suppression of cell viability was minimal with different doses of lidocaine except at a dose of 1000 μM ($P<0.01$; Fig. 1A).

Similarly, apoptosis assay was performed with increasing concentrations of lidocaine (10 μM, 100 μM, and 1000 μM). It was found that lidocaine significantly increased the percentage of apoptotic cells at 100 μM and 1000 μM concentrations ($P<0.05$, and $P<0.005$, respectively; Fig. 1B). This was further confirmed by western blot analysis which revealed that the concentrations of the anti-apoptotic protein Bcl-2 was decreased and those of the pro-apoptotic protein Bax, cleaved caspase-3, and cleaved caspase-9 were decreased (Fig. 1C).

2.2. Lidocaine enhances the sensitivity to 5-FU of SK-MEL-2 cells

5-FU decreased viability of SK-MEL-2 cells in a dose dependent manner, with a significant decrease at 100 μM concentration ($P<0.05$; Fig. 2A). Similarly, treatment with increasing concentrations of 5-FU increased the percentage of apoptotic cells with significant increase at 10 μM and 100 μM concentrations ($P<0.05$ and $P<0.01$, respectively; Fig. 2B). Western blot analysis also

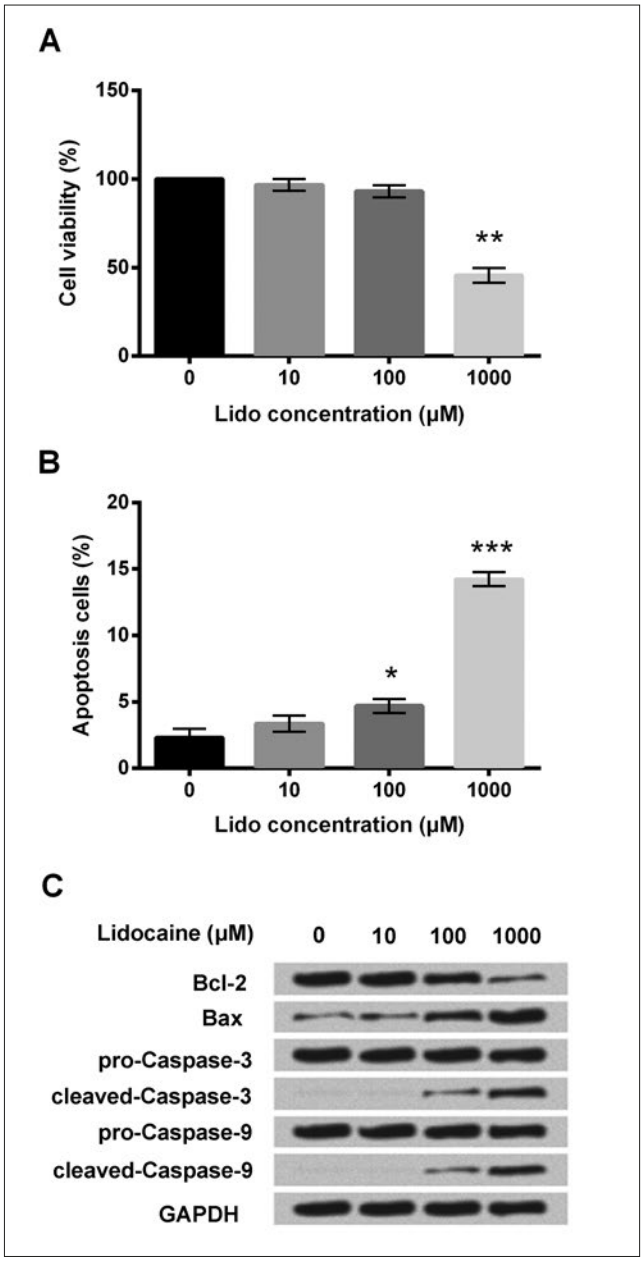

Fig. 1: (A) Effects of different concentrations of lidocaine on cell viability; (B) Effects of different concentrations of lidocaine on apoptosis; (C) Effects of different concentrations of lidocaine on apoptosis by using western blot analysis. *P<0.05; **P<0.01; ***P<0.005.

supported these findings, as concentrations of pro-apoptotic factor Bax, cleaved caspase-3, and cleaved caspase-9 were increased and those of anti-apoptotic factor Bcl2, was decreased following exposure to increasing concentrations of 5-FU (Fig. 2C). Interestingly, increasing concentrations of lidocaine and 5-FU together led to a great decease in cell viability (Fig. 2D) and increase in apoptosis rates (Fig. 2E).

2.3. Lidocaine increases the expression of miR-493

Next, mRNA expression of miR-493 was assessed; it was found that following exposure to increasing concentrations of lidocaine (10 μM and 100 μM), the expression of miR-493 was significantly increased (P<0.05 and P<0.01, respectively; Fig. 3).

2.4. Lidocaine enhances the sensitivity of SK-MEL-2 cells to 5-FU by upregulating the expression of miR-493

Next, mRNA expressions of miR-493 were assessed in SK-MEL-2 cells, transfected with control, NC, or miR-493 inhibitor. It was found that miR-493 inhibitor decreased the miR-493 expression (Fig. 4A).

Assessment of percentage of viable cells revealed that cell viability was significantly decreased (P<0.05) following exposure to both 5-FU (10 μM) and lidocaine (10 μM) compared to the group of cells exposed to only 5-FU (10 μM) (Fig. 4B). However, cell viability was significantly increased (P<0.05) in SK-MEL-2 cells following transfection with miR-493 inhibitor despite exposure to both 5-FU (10 μM) and lidocaine (10 μM) compared to cells without knockdown of miR-493 expression and exposed to both 5-FU (10 μM) and lidocaine (10 μM) (Fig. 4B).

Similarly, apoptosis assay revealed that although apoptosis was significantly increased (P<0.01) in the SK-MEL-2 cells following exposure to both 5-FU (10 μM) and lidocaine (10 μM) compared to the cells treated with only 5-FU (10 μM) (Fig. 4C), knockdown of miR-493 expression in the SK-MEL-2 cells suppressed apoptosis significantly (P<0.05) despite treatment with both 5-FU (10 μM) and lidocaine (10 μM) compared to cells with uninhibited expression of miR-493 and treated with both 5-FU (10 μM) and lidocaine (10 μM) (Fig. 4C). These findings were also supported by Western blot analysis of apoptosis related factors (Fig. 4D).

2.5. miR-493 negatively regulates the expression of Sox4, and Sox4 is a target of miR-493

Next, we assessed the effects of miR-493 expression on expression of Sox. At first with qRT PCR we assessed the expression level of miR-493 in SK-MEL-2 cells following transfection with miR-493 mimics, and miR-493 inhibitor; it was found that the expression of miR-493 was significantly increased (P<0.01) in miR-493 mimic group and significantly decreased (P<0.01) in miR-493 inhibitor group of cells compared to their corresponding control group of cells, (Fig. 5A).

Relative mRNA expression of Sox4 was found to be significantly decreased (P<0.05; Fig. 5B) in SK-MEL-2 cells transfected with miR-493 mimic; similarly, Sox4 expression was significantly increased (P<0.05; Fig. 5B) in SK-MEL-2 cells following transfection with miR-493 inhibitor, compared to their respective control groups of cells. Western blot analysis also supported these findings (Fig. 5C). Thereby it was found that miR-493 negatively affects the expression of Sox4. It was further supported by relative luciferase activity which revealed that expression of Sox-4-wt (wild type) was significantly decreased (P<0.05) in cells transfected with miR-493 (Fig. 5D).

2.6. Overexpression of Sox4 decreases the sensitivity of SK-MEL-2 cells to 5-FU

Full-length Sox4 sequence constructed in pEx-2 and short-hairpin RNA directed against Sox4 constructed in U6/GFP/Neo plasmids were transfected in two different groups of SK-MEL-2 cells, known as pEx-Sox4 and sh-Sox4 groups, respectively. qRT PCR confirmed that the relative mRNA expression of Sox4 was significantly increased (P<0.01; Fig. 6A) in pEx-Sox4 group of cells and was significantly decreased (P<0.01; Fig. 6A) in sh-Sox4 group of cells, compared to their corresponding control group of cells. Western blot analysis also supported these findings (Fig. 6B).

Assessment of cell viability in different groups of cells revealed that cell viability significantly increased (P<0.05; Fig. 6C) despite treatment with both 5-FU (10 μM) and lidocaine (10 μM) in pEx-Sox4 group of cells compared to corresponding control group of cells. Suppression of Sox-4 expression led to a significant suppression of cell viability (P<0.01) compared to the corresponding control group of cells (Fig. 6C). Apoptosis analysis revealed that overexpression of Sox4 significantly suppressed (P<0.05) apoptosis rates despite treatment with both 5-FU (10 μM) and lidocaine (10 μM) compared to the corresponding control group of cells (5-FU (10 μM)+lidocaine (10 μM)+pEx) (Fig. 6D). However, apoptosis was significantly increased in cells with suppression of Sox4 expression and treated with both 5-FU (10 μM) and lidocaine (10 μM) (5-FU (10 μM)+lidocaine (10 μM)+sh-Sox4) compared to corresponding

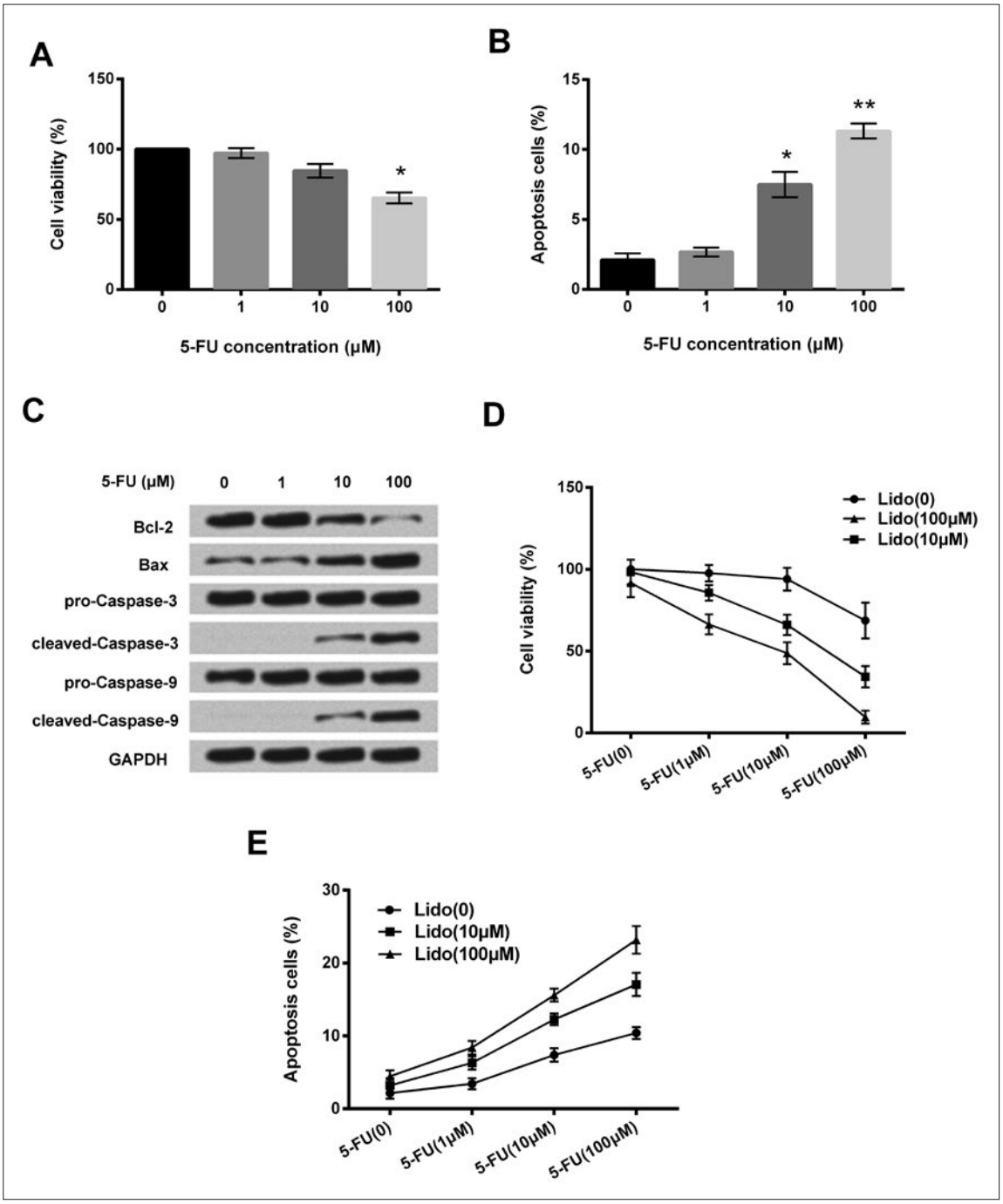

Fig. 2: (A) Effects of different concentrations of 5-FU on cell viability; (B) Effects of different concentrations of 5-FU on apoptosis; (C) Western blot analysis of apoptosis; (D) Cell viability of SK-MEL-2 cells treatment with increasing concentrations of lidocaine and 5-FU. (E) Apoptotic cells of SK-MEL-2 cells following treatment with increasing concentrations of lidocaine and 5-FU. 5-FU: 5-fluorouracil. *P<0.05; **P<0.01.

control group of cells (5-FU (10 μM)+lidocaine (10 μM)+shNC) (Fig. 6D). Western blot analysis of the apoptosis related factors also revealed the same results (Fig. 6E).

2.7. Overexpression of Sox4 in the presence of lidocaine and 5-FU activates the PI3K/AKT and TGF-TGF-β signaling pathways

Western blot analysis of the proteins related to the PI3K/AKT pathway revealed that lidocaine inactivated this pathway; however, overexpression of Sox-4 activated this pathway and vice versa (Fig. 7A). As it was found that concentrations of both p-PI3K and p-AKT were increased in SK-MEL-2 cells overexpressing Sox4 (pEx-Sox4) and treated with lidocaine and 5-FU compared to cells treated with 5-FU, both 5FU and lidocaine, pEX group of cells, and in SK-MEL-2 cells with suppressed Sox-4 activity (sh-Sox4) (Fig. 7A).Again western blot analysis of proteins associated with TGF-β pathway revealed that while lidocaine inactivated this pathway overexpression of Sox-4 activated it (Fig. 7B). As it was revealed that expression of E-cad was decreased and those of vimentin, p-Smad2, and p-Smad3 were increased in SK-MEL-2 cells overexpressing Sox4 (pEx-Sox4) and treated with lidocaine and 5-FU compared to cells treated with 5-FU, both 5FU and lidocaine, pEX group of cells, and in SK-MEL-2 cells with suppressed Sox-4 activity (sh-Sox4) (Fig. 7B).

These results indicate that lidocaine induced the expression of miR-493, and subsequently downregulated the expression of Sox4, enhancing the sensitivity of SK-MEL-2 cells to 5-FU, which might be by inactivation of PI3K/AKT and TGF-TGF-β pathways.

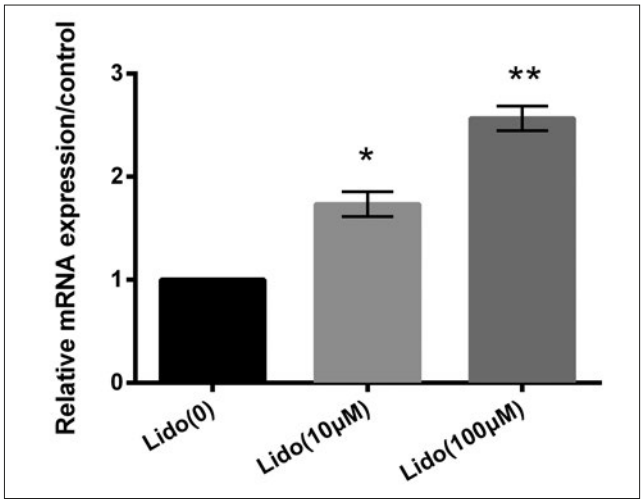

Fig. 3: Relative mRNA expression of miR-493 following treatment with increasing concentrations of lidocaine. *P<0.05; **P<0.01.

3. Discussion

Currently there are number of treatment options for melanoma including surgery, chemotherapy, and immunotherapy (Bataille et al. 2004; Hodis et al. 2012; Russo et al. 2014; Thomas et al. 2006). In the present study, we explored the anti-tumor effects of lidocaine as monotherapy and in combination with 5-FU along with the possible underlying mechanism. We showed that lidocaine suppressed the cell viability (at a concentration of 1000 μM; P<0.01; Fig. 1A) and promoted apoptosis (P<0.05 at concentration of 100 μM and P<0.0001 at a concentration of 1000 μM; Fig. 1B and 1C) in a dose-dependent manner compared to control group of cells not treated with lidocaine.

In a study evaluating effect of lidocaine in breast cancer, combination therapy with lidocaine and bleomycin significantly promoted apoptosis in the human breast cancer cell line MCF-7 compared to monotherapy with either of the drugs (Li et al. 2014). Chlebowski et al. (1982) had already reported that monotherapy with procaine or lidocaine inhibits the growth of SHG cell line (an established human melanoma cell line) similarly to the anticancer drug doxorubicin; additionally, a combination of non-inhibitory doses of procaine and doxorubicin also inhibited the growth of melanoma cells.

We assessed the anti-tumor effects of 5-FU, and found that 5-FU significantly suppressed cell viability (P<0.05; 100 μM; Fig. 2A) and promoted apoptosis (P<0.05 at a concentration of 10 μM; P<0.01 at a concentration of 100 μM; Fig. 2B). Ryan et al. (1988) reported that topical administration of 5-FU converts the infiltrating cells from acute inflammatory stage to T cells in advanced cases of melanoma; they also suggested that pre-operative stimulation of host immunity could improve outcome following surgical resection of the lesions. Several studies have already discussed the anti-cancer effects (suppression of cell viability and promotion of apoptosis of cancerous cells) of 5-FU and lidocaine (Kang et al. 2016; Lirk et al. 2012; Paolino et al. 2008; Ryan et al. 1988). We also assessed the anti-tumor effects of combination of 5-FU and lidocaine at different doses, and found that the combination suppressed cell viability and promoted apoptosis in a dose-dependent manner; maximum benefit was obtained at a dose of 100 μM 5-FU and 100 μM lidocaine (Fig. 2D and 2E).

MicroRNAs are known for their diverse roles in both physiological and pathological conditions (Casey et al. 2016; Takahashi et

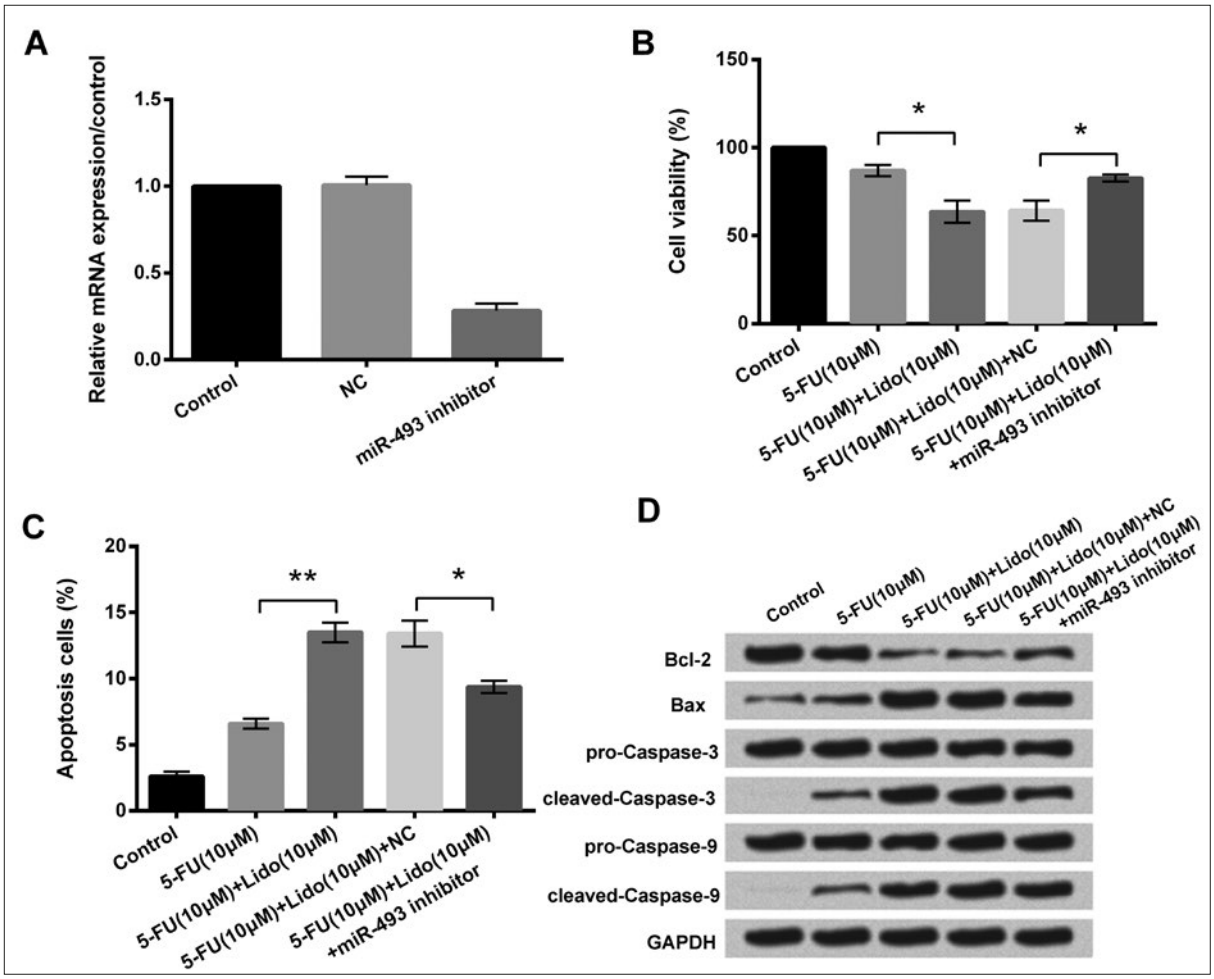

Fig. 4: (A) Relative mRNA expression of miR-493 was decreased in SK-MEL-2 cells transfected with miR-493 inhibitor. (B) Effects of 5-FU (10 μM) and lidocaine (10 μM) with miR-493 inhibitor on cell viability; (C) Effects of 5-FU (10 μM) and lidocaine (10 μM) with miR-493 inhibitor on cell apoptosis; (D) Cell apoptosis by using western blot analysis. *P<0.05; **P<0.01.

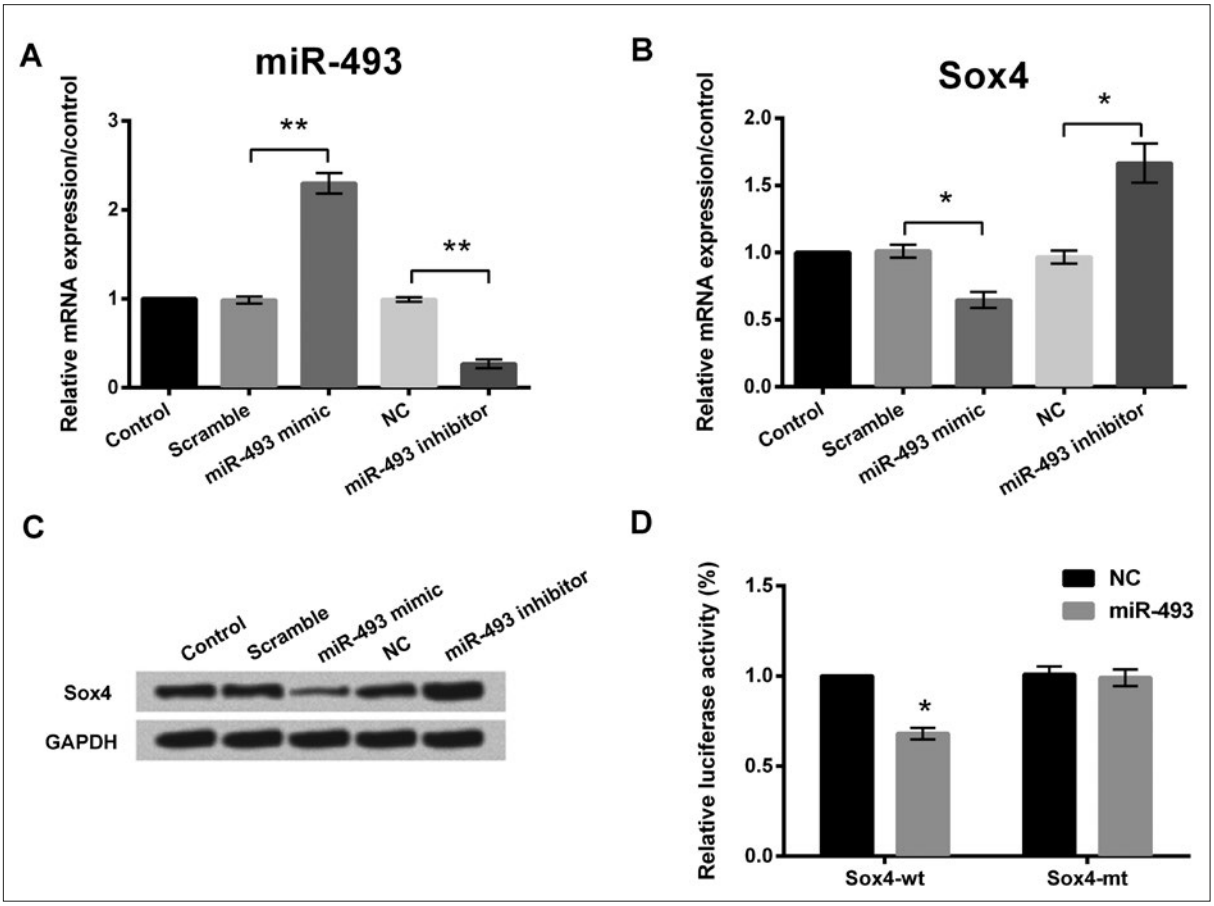

Fig. 5: (A) Relative mRNA expression of miR-493; (B) Relative mRNA expression of Sox4; (C) Relative Sox4 expression by using western blot; (D) Relative luciferase activity of Sox4-wt and Sox4-mt in NC and miR-493. *P<0.05; **P<0.01.

al. 2017). MiR-493 has been known for its anti-tumor effects in different cancers like breast cancer, gastric cancer, colon cancer, lung cancer, etc. (Gu et al. 2014; Sakai et al. 2014; Zhao et al. 2016; Zhou et al. 2015a). To the best of our knowledge, till date, no study has explored the role of miR-493 on the pathogenesis of melanoma In the present study, we found that the relative mRNA expression of miR-493 was significantly increased with increasing concentrations of lidocaine in a dose-dependent manner (P<0.05 and P<0.01 at concentrations of 10 μM and 100 μM lidocaine; Fig. 3). Then we found that lidocaine improves the efficacy of 5-FU by up-regulating the expression of miR-493, as knockdown of miR-493 expression significantly improved cell viability and suppressed apoptosis, despite treatment with combination therapy with 5-FU and lidocaine (P<0.05; Fig. 4B-4D).

We also found that miR-493 suppresses the expression of Sox4 (Fig. 5A-5D). Sox4 is associated with a number of cellular events like embryonic development and cell differentiation (Zhou et al. 2015b). Aberrant expression of Sox4 is reported in a number of cancers; in most of the cancers like lung cancer and colorectal cancer, Sox4 is associated with disease progression (Wang et al. 2016; Zhou et al. 2015b). However, in melanoma, decreased expression of Sox4 is associated with poor prognosis. Jafarnejad et al. (2013) showed that melanoma cells were suppressed due to Sox4 mediated increased expression of Dicer. Similarly, in another study, poor expression of Sox4 was associated with poor prognosis (Jafarnejad et al. 2010). However, in our study overexpression Sox4 was associated with poor sensitivity of the SK-MEL-2 cells to 5-FU and lidocaine combination therapy (Fig. 6C-6E).

Several studies have described the beneficial role of inactivation of PI3K/AKT and TGF-β pathway in cancer, including melanoma (Kou et al. 2016; Medrano 2003). We also found that lidocaine improved the efficacy of 5-FU by upregulating the expression of miR-493 and subsequent suppression of Sox-4 expression in SK-MEL-2 cells by inactivation of the PI3K/AKT and TGF-β pathways (Fig. 7A and 7B).

Thus from the findings of this study it can be concluded that lidocaine enhanced the sensitivity of SK-MEL-2 cells to 5-FU by inducing the expression of miR-493, which negatively regulated the expression of Sox-4; Sox4 was found to inhibit the sensitivity of SK-MEL-2 cells to 5-FU. Additionally, it was also found that lidocaine enhanced the sensitivity of SK-MEL-2 cells to 5-FU by inactivation of PI3K/AKT and TGF-beta pathways.

4. Experimental

4.1. Cell culture and treatment

SK-MEL-2 human melanoma cells were obtained from American Type Culture Collection (Rockville, MD). This cell line was grown in Dulbecco's modified Eagle's medium (DMEM; WelGENE Co., Daegu, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 ˚C under 5% CO_2. The concentrations of lidocaine were 0, 10 μM, 100 μM, and 1000 μM. The concentrations of 5-FU were 0, 1 μM, 10 μM, and 100 μM.

4.2. CCK-8 assay

Cells were seeded in a 96-well plate with 5000 cells/well. Cell proliferation was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, after stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 hour at 37 °C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

4.3. Apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The cells (100,000 cells/well) were seeded in a 6 well-plate. Treated cells were washed twice with cold PBS and re-suspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apop-

totic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

4.4. miRNAs transfection

MiR-493 mimic, miR-493 inhibitor and the negative control (NC) were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

4.5. Transfection and generation of stably transfected cell lines

The full-length Sox4 sequences and short-hairpin RNA directed against Sox4 were constructed in pEX-2 and U6/GFP/Neo plasmids (GenePharma), respectively and were referred to as pEX-Sox4 and sh-Sox4, respectively. The lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) was used for the cells transfection process according to the manufacturer's instructions. The plasmid carrying a non-targeting sequence was used as NC of sh-Sox4 that was referred as to sh-NC. The stably transfected cells were selected by the culture medium containing

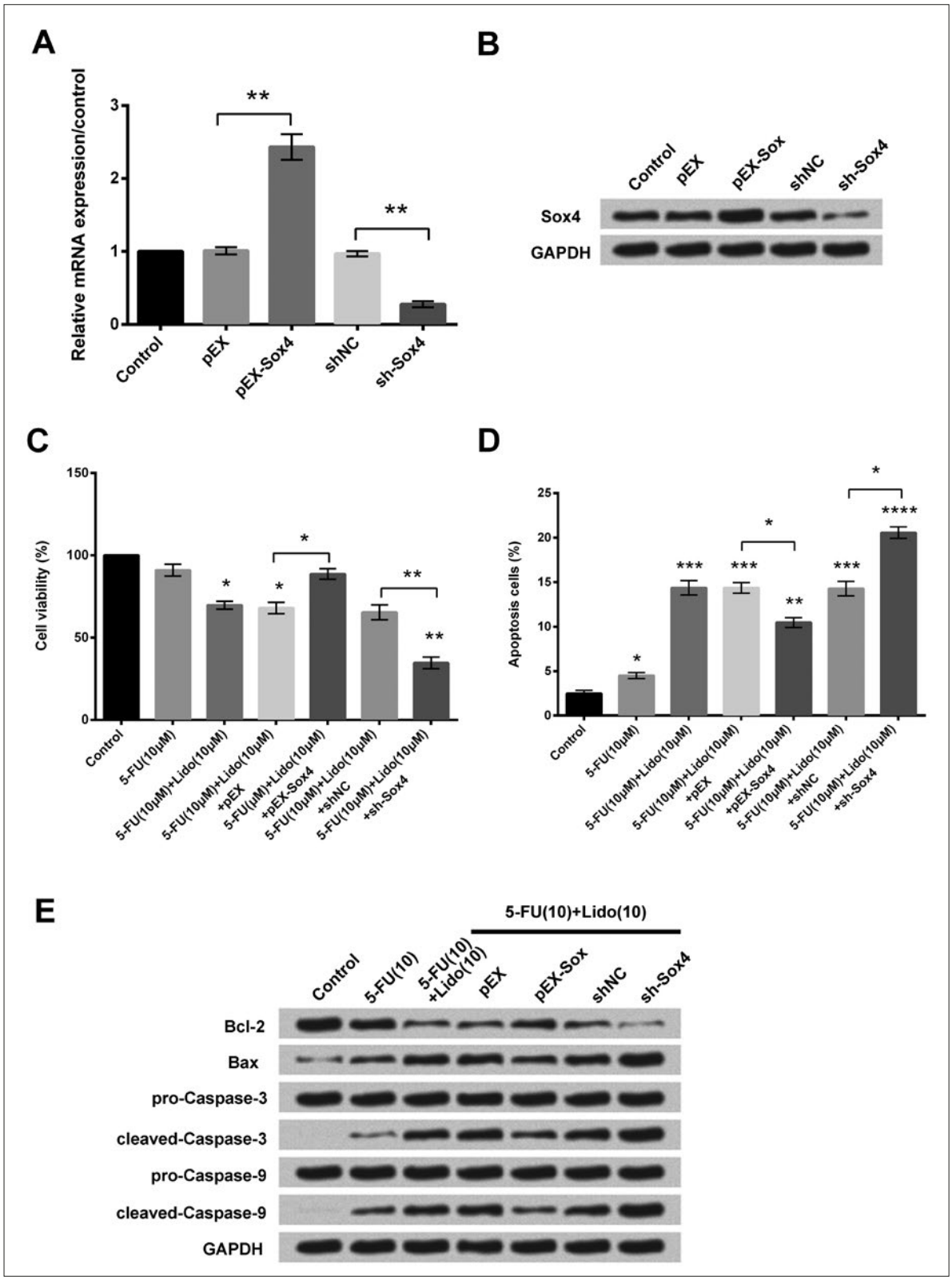

Fig. 6: (A) Relative mRNA expression of Sox4; (B) Relative mRNA expression of Sox4 by using western blot; (C) Cell viability of SK-MEL-2 cells following transfection of the cells with full-length Sox4 sequences treatment with both 5-FU (10 μM), lidocaine (10 μM), U6/GFP/Neo plasmids (sh-Sox4); (D) Cell apoptosis of SK-MEL-2 cells following transfection of the cells with full-length Sox4 sequences treatment with both 5-FU (10 μM), lidocaine (10 μM), U6/GFP/Neo plasmids (sh-Sox4); (E) Cell apoptosis by using western blot. *P<0.05; **P<0.01.

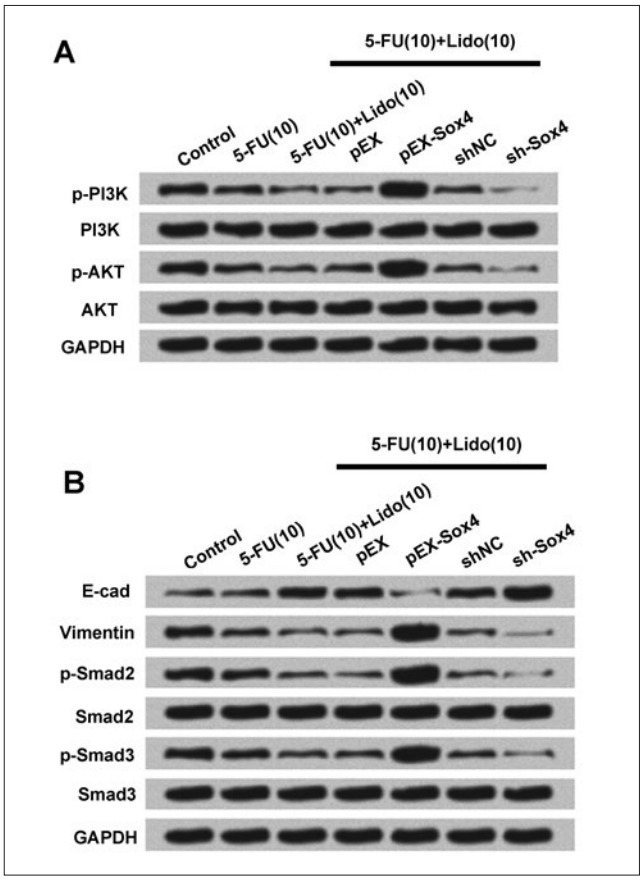

Fig. 7: (A) Western blot analysis revealed that lidocaine enhanced the sensitivity of SK-MEL-2 cells to 5-FU by inactivation of PI3K/AKT pathway as there were decreased expressions of p-PI3K, and p-AKT. (B) Western blot analysis revealed that lidocaine enhanced the sensitivity of SK-MEL-2 cells to 5-FU by inactivation of TGFβ pathway as there were decreased expressions of vimentin, p-Smad2, and p-Smad3.

0.5 mg/ml G418 (Sigma-Aldrich, St Louis, MO, USA). After approximately 4 weeks, G418-resistant cell clones were established.

4.6. qRT-PCR

Total RNA was extracted from cells and tissues using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-493 and U6 (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-493 in cells.

4.7. Dual luciferase activity assay

The 3'UTR target site was generated by PCR and the luciferase reporter constructs with the Sox4 3'UTR carrying a putative miR-493 -binding site into pMiR-report vector were amplified by PCR. Cells were co-transfected with the reporter construct, control vector and miR-493 or scramble using Lipofectamine 3000 (Life Technologies, USA). Reporter assays were done using the dual-luciferase assay system (Promega) following to the manufacturer's information.

4.8. Western Blot

The protein used for western blotting was extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4 °C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

4.9. Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean±SD (standard deviation). Statistical analyses were performed using SPSS 19.0 statistical software. The P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of <0.05 was considered to indicate a statistically significant result.

Conflicts of interest: None declared,

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