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Original Article

Oleanolic acid inhibits aldo-keto reductase family 1 member B10-induced cancer stemness and avoids cisplatin-based chemotherapy resistance via the Snail signaling pathway in oral squamous cell carcinoma cell lines

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KEYWORDS
Aldo-keto reductase family 1 member B10 (AKR1B10);
Oral squamous cell carcinoma (OSCC);
Oleanolic acid (OA);
Oral cancer;
Stemness

Abstract *Background/purpose:* Oral squamous cell carcinoma (OSCC) is a common malignancy often associated with poor prognosis due to chemoresistance. In this study, we investigated whether arecoline, a major alkaloid in betel nuts, can stimulate aldo-keto reductase family 1 member B10 (AKR1B10) levels in OSCC, promoting cancer stemness and leading to resistance to cisplatin (CDDP)-based chemotherapy.
Materials and methods: Gain- and Loss- of AKR1B10 functions were analyzed using WB and q-PCR of OSCC cells. Stemness, epithelial mesenchymal transition (EMT) markers, and CDDP drug resistance in overexpressed AKR1B10 were also identified.
Results: Upregulated AKR1B10 in OSCC significantly increased cell motility and aggregation. The results also showed that the canonical TGF-β1-Smad3 pathway was involved in arecoline-induced AKR1B10 expression, further increasing cancer stemness with CDDP

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resistance via the Snail-dependent EMT pathway. Moreover, oleanolic acid (OA) and ROS/RNS (reactive oxygen/nitrogen species) inhibitors effectively reversed AKR1B10-induced CDDP-resistance.

Conclusion: Arecoline-induced ROS/RNS to hyper-activate AKR1B10 in tumor sphere cells via the TGF-β1-Smad3 pathway. Furthermore, AKR1B10 enhanced CDDP resistance in OSCC cells via EMT-inducing markers. Finally, Finally, OA may efficiently target CDDP resistance, reverse stemness in OSCC cells, and have the potential as a novel anticancer drug.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide.[1,2] Both the incidence and mortality of oral cancer rank fourth among the top ten cancers in men in Taiwan. The number of new oral cancer cases and the number of patients who died of oral cancer in Taiwan in 2020 were 8277 and 3,380, respectively. Notably, owing to high locoregional recurrence, the 5-year survival rate has remained at approximately 20—25 % for advanced-stage OSCCs over the last decade in Taiwan.[3]

Accumulating evidence has indicated that cancer stem cells (CSCs) contribute to high rates of locoregional recurrence and drug resistance because of their ability to self-renew and proliferate tumor cells[4] as well as remain in a quiescent G0 state to avoid being destroyed by radiotherapy (RT) and chemotherapy.[5] In addition, the crosstalk between the tumor microenvironment (TME) and CSCs enhances the evasion of immune surveillance via camouflage by reducing MHC class I (MHC-I) molecules while escalating angiogenesis.[6]

Cisplatin-based (cis-diamminedichloroplatinum, CDDP) chemotherapy has long been the first-line treatment for head and neck cancer in combination with targeted medication and immunotherapy, in addition to surgical resection and RT.[7,8] In general, CDDP exerts a tumoricidal effect on cancer cells via 2 main mechanisms. First, CDDP enables covalent binding to native DNA in linear and circular forms, thus altering DNA structures, interaction with which can produce numerous CDDP-DNA adducts that inhibit DNA replication and chain elongation. This has been reported as the main cause of antineoplastic activity.[9,10] Second, CDDP-related cytotoxicity also generates abundant reactive oxygen species (ROS) in mitochondria, thereby disturbing electron transport chains, leading to the collapse of energy production, which finally results in the activation of programmed cell death such as apoptosis.[11,12]

Nevertheless, the emergence of drug resistance presents major challenges for cancer treatment, including decreased drug uptake, increased drug efflux, alterations in the drug target, drug metabolism, repair of DNA damage and cell cycle checkpoint mediators, and changes in the downstream mediators of the apoptotic pathway.[13] Furthermore, recent studies have also demonstrated that the TME plays a key role in the development of CDDP resistance[14] through metabolic plastic adaptation under adverse conditions such as hypoxia, acidosis, and starvation[15] as crucial regulators of cancer hallmarks to achieve stemness, invasion, metastasis, and resistance to anticancer therapies.[16—18]

Aldo-keto reductase family 1 member B10 (AKR1B10), also known as aldose reductase-like-1, is a metabolic enzyme that efficiently converts cytotoxic and carcinogenic carbonyl compounds, including reactive aldehydes, retinoids, and isoprenoids, into less toxic hydroxyl groups.[19] Studies have proposed that AKR1B10 is implicated in cell proliferation, carcinogenesis, and cancer therapeutics through xenobiotic detoxification, regulation of retinoic acid levels, cellular fatty acid synthesis and lipid metabolism.[20—22] We found that gene transcription of AKR1B10 in SAS sphere cells (SASsp) was significantly higher than in parental cells in terms of lipid metabolism and ROS scavengers. In addition, we demonstrated[23,24] that higher AKR1B10 levels in tissue and saliva were significantly associated with larger tumor size, advanced clinical stage, areca quid chewing, and poor survival in OSCCs. Elevated expression of AKR1B10 has also been found in a wide variety of human tumors, such as hepatocellular,[19] non-small cell lung,[23] breast,[24] cervical,[25] and OSCC.[26,27]

In this study, we investigated whether arecoline, a major alkaloid in betel nuts, could stimulate aldo-keto reductase family 1 member B10 (AKR1B10) levels in OSCC, which in turn promoted cancer stemness and led to resistance to cisplatin (CDDP)-based chemotherapy. Thus, the main objective of this study was to verify whether arecoline can raise AKR1B10 expression to promote proliferation and sphere formation in OSCC cells. We also aimed to elucidate whether the TGF-β1 (transforming growth factor-beta) pathway was involved in arecoline-induced AKR1B10 expression, further increasing cancer stemness via epithelial mesenchymal transition (EMT) markers. Finally, we investigated whether oleanolic acid (OA), an AKR1B10 inhibitor, could block CDDP resistance and have a potential as a novel anticancer drug.

Materials and methods

Materials

Human TGFβ1 was obtained from R&D Systems (R&D Systems, Minneapolis, MN, USA). N-acetyl-L-cysteine (NAC)-universal ROS inhibitor, Trolox- ROO- inhibitor, L-nitro-arginine methyl ester hydrochloride (L-NAME)- NO (Nitric

oxide) inhibitor, uric acid- ONOO (Peroxynitrite).- inhibitor, mannitol- HO. inhibitor, MitoTempo- mitochondria-targeted antioxidant, allopurinol- xanthine oxidase-dependent ROS inhibitor, plumbagin- NADPH oxidase 4 inhibitor, and OA- AKR1B10 inhibitor were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Corp, St. Louis, MO, USA.), and diphenyleneiodonium chloride (DPI)- NADPH oxidase inhibitor and apocynin- NADPH oxidase 2 inhibitor were purchased from Merck Calbiochem (Merck & Co., Inc., Kenilworth, NJ, USA).

Cell culture

The human OSCC cell lines SAS, Ca9-22, and HSC-3 were provided by the Japanese Collection of Research Bioresources cell bank. Cells were plated on 60-mm Petri dishes at a density of 2×10^5 cells and subjected to various treatments, as previously described.[28] The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (Corning, NY, USA), 10 % heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin (Corning, NY, USA) at 37 °C in a humidified atmosphere containing 5 % CO_2. To examine the effects of arecoline (Sigma 31593, Sigma-Aldrich Co.) and TGF-β1 (R&D Systems) on OSCC cells, equal numbers of SAS, Ca9-22, and HSC-3 cells were plated in serum-free medium, starved for 16 h (or overnight), and then treated with arecoline (0.2 mM) or TGF-β1 (2 ng/mL) for 0—3 days.

Overexpression of AKR1B10 and siRNA AKR1B10 transfection cells

To incorporate overexpressed AKR1B10 plasmids into SAS and Ca9-22 cells, a pCMV6-AC-GFP construction carrying AKR1B10 (RG203177, OriGene, Rockville, MD, USA) was transfected into OSCC cells by using Lipofectamine 3000 Transfection reagent (Invitrogen, Carlsbad, CA, USA). SAS and Ca 9—22 cells were transfected overnight and the culture medium was replaced with a selection culture medium containing 1—2 g/L of G418 (InvivoGen, San Diego, CA, USA). After subculturing, the overexpressed AKR1B10 cells were selected using a cell sorting cytometer (BD FACSAria, Franklin Lakes, NJ, USA) and then maintained in medium containing 0.4 g/L of G418. Human AKR1B10 siRNA was purchased from Santa Cruz Biotechnology, Inc. (sc-72341, Santa Cruz, CA, USA). For transient knockdown expression, reverse transfection combined with forward transfection was used to transfect 5×10^5 cells in each 6-well plate with Lipofectamine™ RNAiMAX (Invitrogen).

Colony forming assay

Human OSCC cells (500, 1000, and 2000) were seeded in 6-well plates. After incubation for 10—14 days at 37 °C in 5 % humidified CO_2, the cells were washed with phosphate-buffered saline (PBS) and stained with 0.5 % crystal violet in methanol for 5—10 min at room temperature. Cells were washed with PBS twice and visible colonies were counted.

Cell migration and invasion assay

A Boyden chamber assay was used to evaluate cell migration and invasion with 8.0-μm ThinCert inserts (Greiner Bio-One, Monroe, NC, USA) in a 24-well plate. Cells (5×10^4) with serum-free DMEM seeded in the upper layer and 2 % fetal bovine serum medium in the lower layer were used to study the migration activity of OSCC cells. Inserts coated with 3 mg/mL of Matrigel Matrix (BD Biosciences, San Jose, CA, USA) were used to study the invasion activity of OSCC cells. An OSCC cell suspension (1×10^5) in serum-free DMEM was seeded in a Matrigel Invasion Chamber. After 24 or 48 h of cultivation, invasive cells on the underside of the chamber were fixed with 100 % methanol, stained with 1 % crystal violet, and observed under a high-power field microscope. Invasive cells on the membrane were lysed using 10 % acetic acid, and the optical density of the crystal violet released in the cells at 570 nm was determined using a microplate reader.[29]

Sphere culture and sphere forming assay

A 10-cm dish with 2×10^5 OSCC cells was pre-coated with 1 % agarose gel (Infinigen Biotec, Green Dr City of Industry, CA, USA).

Western blotting

OSCC cells were seeded on 6-well plates at a density of 2×10^5/plate. After 2 days of growth, cells were drug-treated, collected, and lysed in radioimmunoprecipitation assay buffer containing IX Protease Inhibitor Cocktail (MedChem Express, Monmouth Junction, NJ, USA). Samples were denatured with Tris-Glycine SDS sample buffer, boiled at 95 °C for 10 min, separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes (BioTrace™ NT, Pall Corporation, Washington, NY, USA). Non-specific binding was blocked via incubation in 5 % (w/v) non-fat milk in tris-buffered saline (TBS) for 1 h at room temperature. Anti-human primary antibody was incubated at 4°C overnight. After washing with TBS with Tween, horseradish peroxidase-conjugated secondary antibody was incubated at room temperature for 1 h. Chemiluminescence was detected using a LAS-4000 system (Fujifilm Life Science, Cambridge, MA, USA).

Cisplatin cytotoxicity test

Cisplatin (Kemoplat Inj 0.5 mg/mL, Fresenius Kabi, Bad Homburg, Germany) cytotoxicity was analyzed using a 1-mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich Co.). Cells were seeded on 24-well plates (2.5×10^4 per well) and treated with various concentrations of cisplatin for 48 h. The MTT test was then conducted and absorbance at 570 nm was measured. Cell viability was calculated using the following formula: [OD sample / OD blank control] × 100 %. Triplicate experiments were executed and average values with mean ± standard error of the mean (SEM) were obtained.

Statistical analysis

The data were presented as means ± SEM. Differences between AKR1B10 expression and clinicopathological parameters were analyzed using a 2-tailed Student's *t*-test. We used the Kaplan—Meier product-limit method and Cox regression model to analyze cumulative survival (Statistica version 7, StatSoft, Tulsa, OK, USA). A *P*-value of <0.05 was considered statistically significant.

Results

AKR1B10 promotes proliferation, migration, and colony formation in OSCC cells

To investigate the lipid metabolism and ROS/RNS (reactive oxygen/nitrogen species) scavenger genes associated with stemness, we compared the gene transcripts of parental SAS with those of SASsp by using Affymetrix microarray analysis. The results showed that AKR1B10 had the highest expression in the heatmap of several candidate genes (Fig. 1A). In further investigation of the gene expression of AKR1B10 between parental SAS and SASsp, the results confirmed significantly higher expression of AKR1B10 in SASsp according to Western blot analysis (Fig. 1B, left panel) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 1B, right panel). After building stable OSCC cell lines and detecting endogenous AKR1B10 expression, we found higher expression of AKR1B10 in HSC-3 but lower expression in SAS, Ca9-22, and other oral cell lines (Fig. 1C). Overexpressed AKR1B10, revealed by Western blotting (Fig. 1D, upper panel) with plasmid transfection, was found to promote proliferation using pCMV6-AC-GFP in SAS and Ca9-22 (Fig. 1D, middle and lower panels). Furthermore, transfected AKR1B10 in SAS and Ca9-22 cell lines significantly increased cell motility in the Transwell migration assay (Fig. 1E) and showed higher cell aggregation in the colony forming assay than wild type cells (Fig. 1F). By contrast, knockdown of AKR1B10 in SAS and HSC-3 cells significantly attenuated the migration ability (Fig. 1G).

Arecoline stimulates AKR1B10 synthesis by activating the TGF-β1 pathway

Investigation of whether arecoline induced AKR1B10 expression revealed that AKR1B10 was upregulated in SG cells in a time-dependent manner (Fig. 2A). Moreover, arecoline stimulated significantly higher secretion within 15—30 min in SAS and Ca9-22 cells (Fig. 2B). However, TGF-β-neutralizing antibody SB431542 and SIS3 significantly attenuated arecoline-induced AKR1B10 synthesis (Fig. 2C). Furthermore, we examined the canonical Smad3 pathway in the TGF-β1 signaling pathway. As shown in Fig. 2D,

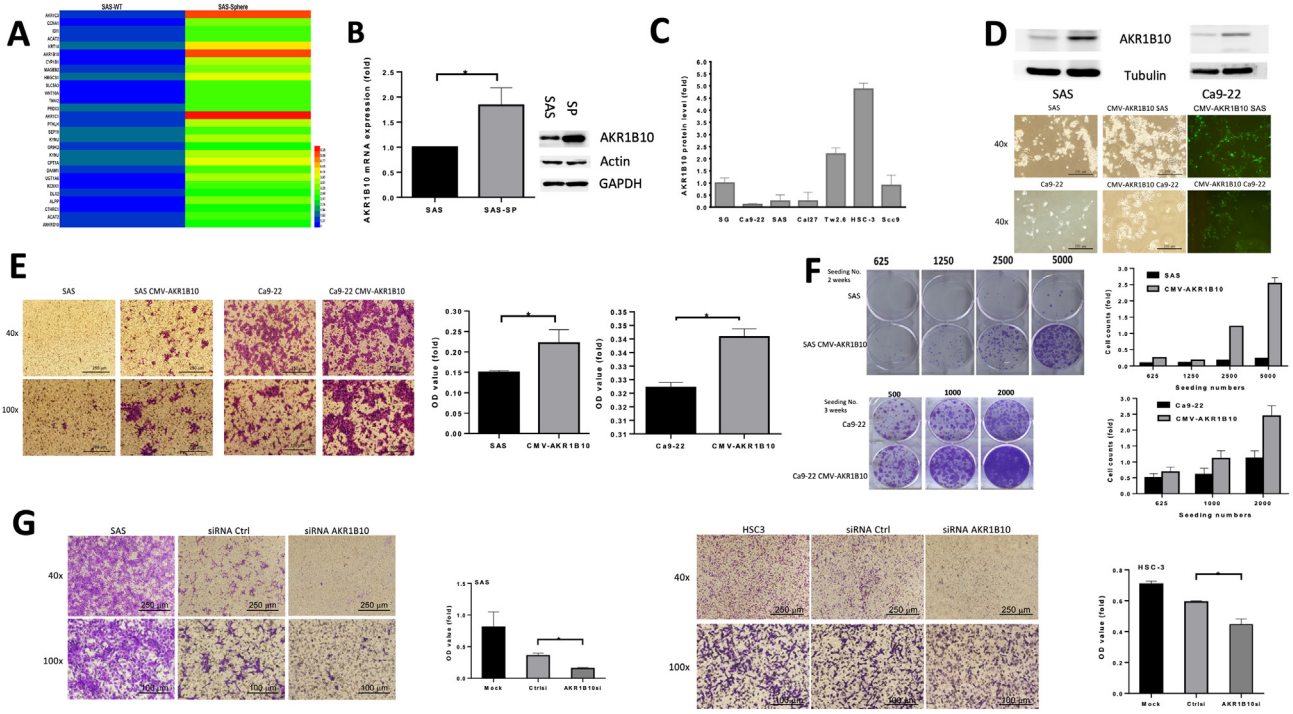

Figure 1 'The overexpressed AKR1B10 in SASsp. (A) The heatmap of the metabolic microarray analysis showed that AKRIB10 was significantly upregulated in SASsp compared to that in parental SAS cells. (B) Overexpressed AKR1B10 in Western blot analysis (left panel) and qRT-PCR (right panel). (C) Higher expression of AKR1B10 in the HSC-3 cell line but lower expression in SAS, Ca9-22, and other oral cell lines. (D) Overexpressed AKR1B10 in Western blot analysis (upper panel) based on plasmid transfection was found to promote proliferation using pCMV6-AC-GFP in SAS and Ca9-22 (middle and lower panels). (E) Transfected AKR1B10 in SAS and Ca9-22 cell lines significantly increased cell motility in the Transwell migration assay, and (F) showed higher cell aggregation in the colony forming assay than that in wild type cells. (G) Knockdown of AKR1B10 in SAS and HSC-3 cells significantly attenuated migration ability.

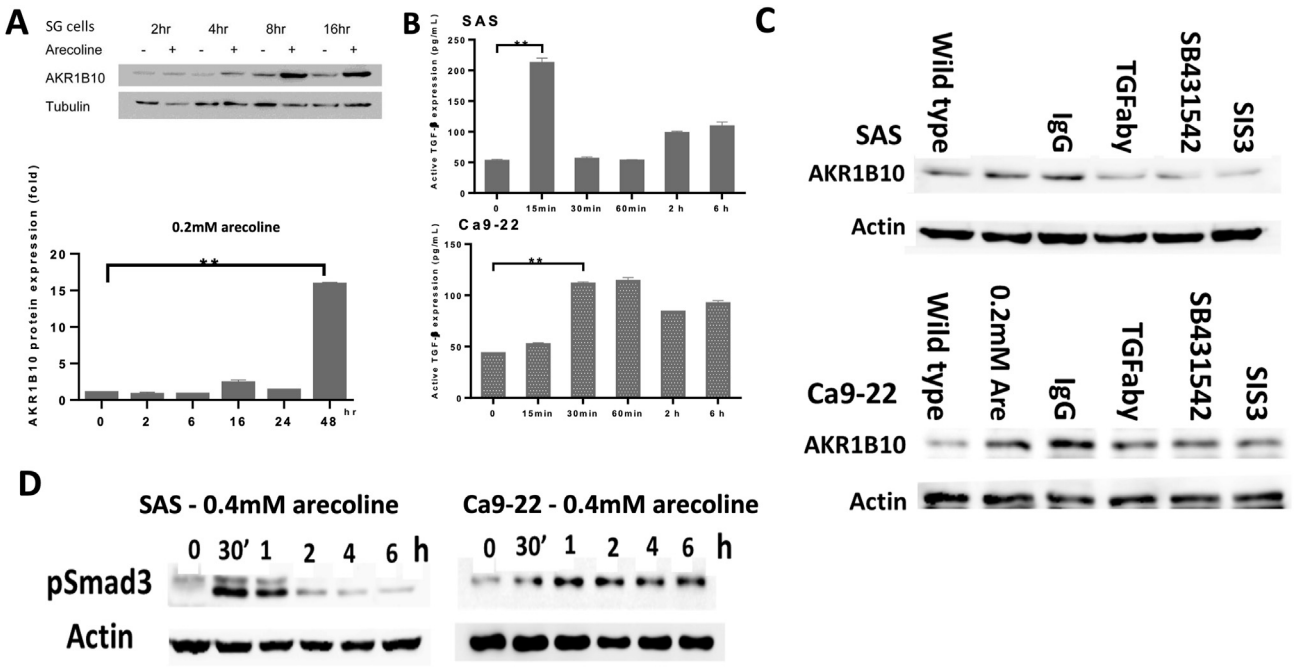

Figure 2 Arecoline induced AKR1B10 synthesis by activating the TGF-β1-Smad3 pathway. (A) Arecoline induced AKR1B10 expression in SG cells in a time-dependent manner (upper panel) and in SAS cells (lower panel). (B) Arecoline stimulated higher TGF-β1 secretion according to Western blotting within 15—30 min in SAS and Ca9-22 cells. (C) TGF-β1 upregulated the downstream synthesis of AKR1B10. (D) Arecoline significantly induced Smad3 phosphorylation after 1 h of exposure.

arecoline significantly induced Smad3 phosphorylation after 30 min to 1 h. We then examined the effects on arecoline-induced AKR1B10 protein synthesis; these data indicated that arecoline stimulated AKR1B10 synthesis in SAS and Ca9-22 cells by activating the TGF-β1-Smad3 pathway.

AKR1B10 increases cancer stemness via the Snail-dependent EMT pathway in SAS and Ca9-22

Next, we found significantly higher sphere colony formation in SAS CMV-AKR1B10 and Ca9-22 CMV-AKR1B10 than in parental SAS and Ca9-22 cells (Fig. 3A). By contrast, knockdown of AKR1B1-SAS cells significantly blocked the colony formation ability in a dose-dependent manner (Fig. 3B). In addition, overexpressed AKR1B10 was significantly correlated with stemness markers, including Nanog, OCT4, and SOX2 in SAS and Ca9-22 cells (Fig. 3C). Similarly, the expression of stemness markers was significantly attenuated by knockdown of AKR1B10 cells (Fig. 3D). Furthermore, we found that upregulated AKR1B10 increased EMT markers such as Snail, Slug, and Twist, and also noted downregulated expression in EMT markers after the knockdown of AKR1B10 (Fig. 3E). The results suggested that AKR1B10 increases cancer stemness via the Snail pathway in oral cancer.

OA and ROS/RNS inhibitors block AKR1B10-induced cisplatin resistance

We established stable clones of SAS/Ca9-22 overexpressing AKR1B10 to detect the effect on CDDP sensitivity. Western blot analysis of AKR1B10 in SAS-CMV-AKR1B10/Ca9-22-CMV-AKR1B10 cells treated with CDDP revealed that the upregulated expression of AKR1B10 enhanced CDDP resistance in a dose-dependent manner (Fig. 4A, left panels). Analysis of the effect of a 50 % lethal dose (LD_{50}) of CDDP with or without CMV-AKR1B10 transient transfection revealed a significantly higher LD_{50} of CDDP for AKR1B10-transfected SAS/Ca9-22 clones than for those without AKR1B10 transfection (Fig. 4A, right panels). Analysis of the efficacy of OA[30] as a selective inhibitor of AKR1B10 revealed that the overexpression of AKR1B10 significantly reduced the cytotoxicity of CDDP (Fig. 4B, upper panels). Moreover, the silencing of AKR1B10 expression via pretreatment with OA at different doses reduced the stemness marker expression in SAS cells (Fig. 4B, lower panels). Furthermore, we examined whether ROS/RNS were involved in the AKR1B10-induced CDDP resistance by activating the TGF-β1 pathway. Significant reduction in the AKR1B10 protein by ROS/RNS inhibitors such as NAC, L-NAME, Trolox, mannitol, and uric acid (Fig. 4C, upper panel) was noted, as well as significant attenuation in the production of active TGF-β1 (Fig. 4C, lower panel). However, MitoTempol, DPI, apocynin, and phenylbiguanide had no effect on the development of TGF-β1 and AKR1B10. The schematic diagram in Fig. 4D shows that arecoline and CDDP induced overexpression of the AKR1B10 protein via ROS/RNS production, activating the canonical TGF-β1-Smad3 pathway and in turn provoking downstream stemness through signaling of the Snail-dependent EMT pathway. Nevertheless, OA and ROS/RNS inhibitors effectively blocked AKR1B10-induced CDDP resistance (Fig. 4D).

Discussion

In this study, we first identified that the AKR1B10 protein was significantly upregulated in SASsp cells, and

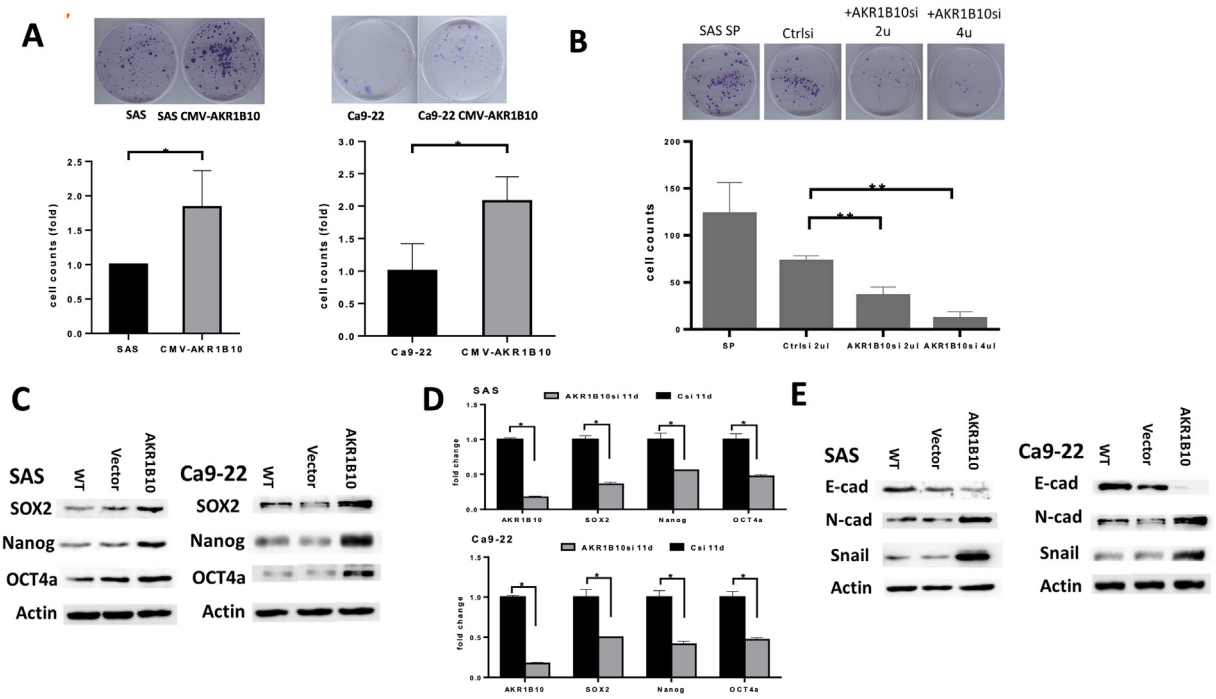

Figure 3 AKR1B10 increased cancer stemness via the Snail signaling pathway. (A) Significantly higher sphere colony formation was found in SAS CMV-AKR1B10 and Ca9-22 CMV-AKR1B10 than in parental SAS and Ca9-22 cells. (B) Knockdown of AKR1B1-SAS cells significantly blocked colony formation in a dose-dependent manner. (C) Overexpressed AKR1B10 was significantly correlated with stemness markers, including Nanog, Oct4, and SOX2, in 2 cell lines. (D) The expression of stemness markers was significantly attenuated by siAKR1B10 cell lines. (E) Upregulated AKR1B10 increased EMT markers such as Snail, Slug, and Twist as well as downregulating expression in EMT markers after the knockdown of AKR1B10.

subsequently demonstrated the enhancement of functional abilities via the gain- and loss-of-function of the *AKR1B10* gene in SAS and Ca9-22 cell lines. Moreover, the canonical TGF-β1-Smad3 pathway was involved in arecoline-induced AKR1B10 protein expression, further increasing cancer stemness with CDDP resistance via the Snail-dependent EMT pathway. Furthermore, OA and ROS/RNS inhibitors effectively reversed AKR1B10-induced CDDP-resistance in SAS and Ca9-22 cell lines. The results further address our previous clinical data on tissue and saliva.[26,27,31]

Mechanistically, AKR1B10 is involved in fatty acid synthesis via regulation of acetyl-CoA carboxylase α (ACCA) stability through the degradation of ubiquitin.[32] Overexpressed lipogenic enzymes such as ACCA and fatty acid synthase are reported to correlate with breast, liver, prostate, and oral cancers.[30,33—35]

In addition to regular energy production from aerobic glycolysis, fatty acid oxidation has been suggested to provide adequate energy demands, tumor proliferation, and chemoresistance, as well as increasing the phenotype of CSCs in favorable TMEs.[36] Therefore, reasonable speculation suggests that upregulated AKR1B10 may result in metabolic adaptation in TMEs, benefitting CSC survival via enhanced ACCA stability and fatty acid synthesis.

Recent studies have disclosed that upregulation of AKR1B10 expression may play a critical role in the formation of chemoresistance against cyclophosphamide and Mitomycin-c in medulloblastoma D341 and colon cancer HT29 cells.[37,38] Furthermore, AKR1B10 has been reported to develop CDDP resistance in gastrointestinal cancer cells through a inhibitory PPAR-γ-dependent mechanism.[28] However, the tumoricidal effect of these chemotherapeutic drugs was partially to generate ROS to destroy nucleic acids, proteins, and lipids to form highly toxic carbonyl compounds to kill cancer cells. One of the mechanisms of AKR1B10 is the effective reduction of aldehydes through lipid peroxidation into alcohols to produce chemoresistance in tumor cells.[39] Moreover, the cellular levels of oxidants modulate their functions via redox homeostasis. Nitric oxide (•NO), a free radical and oxidant, represents a major physiological regulator of CDDP resistance in human lung cancer cells through the activation of the Nrf2/Keap1 and NF-κB pathways to upregulate AKR1B10.[40—43] ROS/RNS function as key regulators that mediate AKR1B10 expression in terms of CDDP resistance.

Next, we found that arecoline-induced ROS/RNS activated AKR1B10 via canonical the TGF-β1-smad3 pathway in SAS and Ca9-22. ROS/RNS are produced during areca quid chewing, inducing an inflammatory reaction and recruiting inflammatory cells to secrete pro-inflammatory cytokines such as TGF-β1, TNF-α, IL-1, IL-6, and IL-12 into the oral mucosa.[44] TGF-β1 signaling is complicated but of paramount importance to the regulation of cell cycle arrest and apoptosis in early-stage tumors, in contrast to triggering uncontrolled cell proliferation against apoptosis in late-stage tumors.[45] Therefore, TGF-β1 can act like a double-edged sword; when the carcinogenic effect surpasses the beneficial effect, oral cancer is finally formed. In addition,

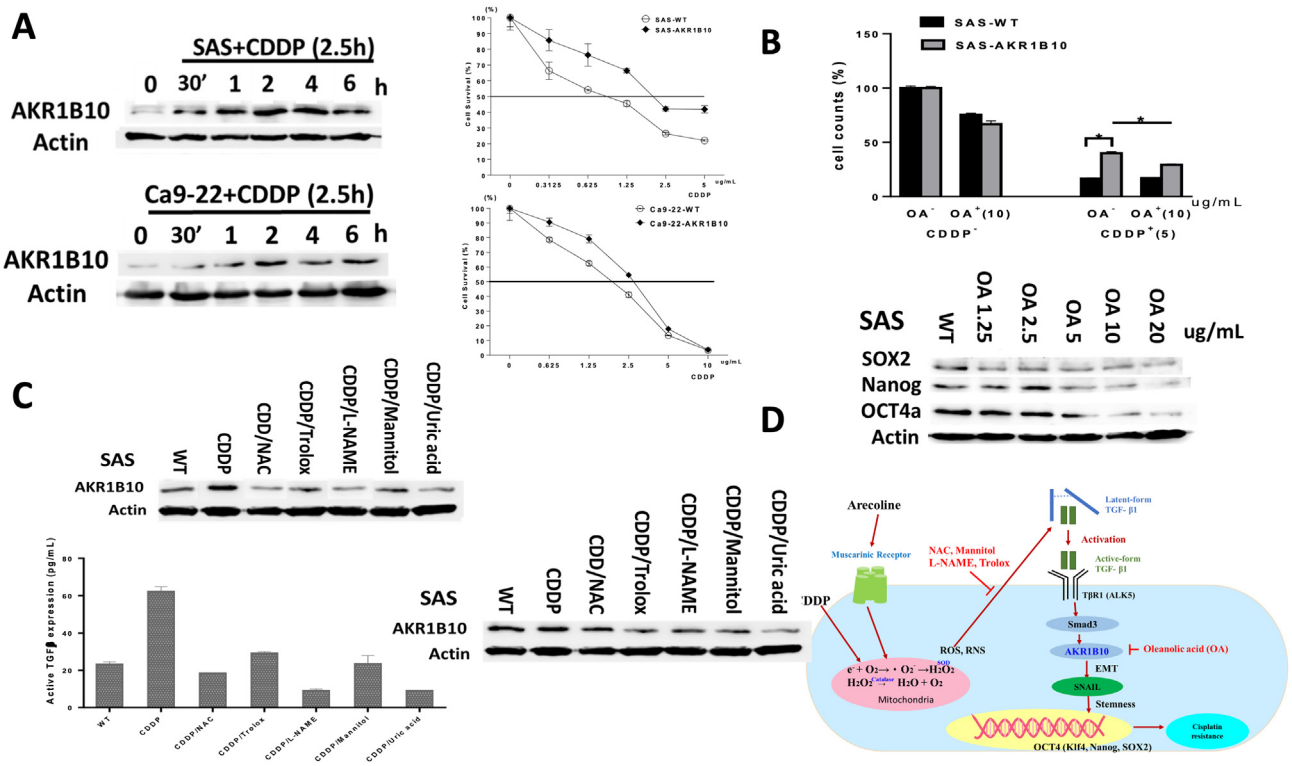

Figure 4 OA and ROS/RNS inhibitors blocked AKR1B10-induced CDDP resistance. (A) SAS/Ca9-22 cell lines were cloned with stable overexpression of AKR1B10 to detect the effect on CDDP sensitivity. Western blot analysis of AKR1B10 in SAS-CMV-AKR1B10/Ca9-22-CMV-AKR1B10 cells treated with CDDP revealed that upregulated expression of AKR1B10 enhanced CDDP resistance in a dose-dependent manner (right panels). Significantly higher LD_{50} induced by CDDP was noted in AKR1B10-transfected SAS/Ca9-22 clones than in those without AKR1B10 transfection (left panels). (B) Analysis of the efficacy of OA as a selective inhibitor of AKR1B10 revealed that the overexpression of AKR1B10 significantly reduced the cytotoxicity of CDDP (upper panels). Moreover, silencing of AKR1B10 expression through pretreatment with OA at different doses reduced the stemness marker expression in SAS/Ca9-22 cells (lower panels). (C) The AKR1B10 protein was significantly reduced by ROS/RNS inhibitors such as NAC, L-NAME, Trolox, mannitol, and uric acid (upper panel), as well as significant attenuation in the production of active TGF-β1 (lower panel). (D) A schematic diagram showing arecoline and CDDP inducing overexpression of the AKR1B10 protein via ROS/RNS production, activating the canonical TGF-β1-Smad3 pathway and in turn provoking downstream stemness through signaling Snail excitation. Nevertheless, OA and ROS/RNS inhibitors effectively blocked AKR1B10-induced CDDP resistance.

TGF-β1 is involved in the pathway of EMT-induced cancer stemness via potent TGF-β1 and Wnt autocrine signaling, thereby contributing to CSC properties.[46] We found that TGF-β1 promoted downstream AKR1B10 secretion but down-regulated it via TGF-β1-neutralizing antibody, TβRI inhibitor-TSB431542, and Smad3 inhibitor-SIS3. The canonical TGF-β1-Smad3 pathway was suggested to be involved in affecting AKR1B10 production in OSCC cell lines.

One study demonstrated that AKRB10P1 (Pseudogene AKR1B10) promoted tumor proliferation and mediated EMT in hepatocellular carcinoma through SOX4 stabilization. Depletion of AKR1B10P1 diminished EMT markers in N-cadherin and Vimentin but this was reversed in E-cadherin.[47] By contrast, AKR genes including AKR1B1/AKR1B10 were abnormally expressed in colon cancer, and positively correlated with AKR1B1 as well as negatively correlated with AKR1B10 in disease free survival and OS, respectively. In addition, AKR1B1/AKR1B10 was positively and negatively related to the EMT markers ZEB, Twist, and Vimentin.[48] Nevertheless, we found that AKR1B10 was significantly upregulated in tissue, saliva, and serum in OSCCs, and activated the production of Snail, Twist, and Slug in EMT-inducing transcription factors (EMT-TFs). These disparities may produce distinct organ-specific carcinogenesis such as easily inflamed mucosa irritated by normal oral flora and high-risk oral habits, particularly areca quid chewing. EMT is characterized by epithelial cells losing polarity and adhesion, converting to a mesenchymal phenotype, and in turn facilitating tumor cell migration, invasion, and metastasis.[49] Moreover, EMT likely contributes to phenotypic differences between CSCs and non-CSCs,[50] which are known to acquire CSC properties through the TGFβ-Smad and Wnt-β-catenin pathways via autocrine signaling loops,[51] and activate adenosine triphosphate-binding cassette transporter genes to efflux chemotherapy drugs outside the cell.[52] Furthermore, CSC markers such as Nanog, Oct4, and SOX2 are positively up-regulated under AKR1B10 stimulation, in contrast to silencing of AKR1B10, which clearly reduces the levels of Nanog, Oct4, and SOX2. AKR1B10 is positively modulated by upstream canonical TGF-β1-Smad3, and in turn acquires chemoresistance by activating the EMT-CSC pathway.

From an oncological perspective, prenylation has attracted more attention to AKR1B10 because of the

activation of the proto-oncogene Ras protein. Hydrophilic Ras protein requires prenylation in c-terminal cysteine residue by AKR1B10 via farnesyltransferase for stable anchoring in the cell membrane.[53] Silencing AKR1B10 expression in pancreatic cancer cell lines significantly diminishes membrane-bound prenylated K-Ras protein, E-cadherin, and downstream phosphorylated ERK.[54] Previously, we have shown that up to 92.2 % of *ras* p21-positive immunostaining was correlated with patients' daily or total areca quid consumption in OSCC patients.[55] Moreover, arecoline stimulates TGF-β1 and phosphorylated-EGFR has been found to provoke the downstream Ras/Erk MAPK pathway in SAS and Ca9-22 cells (data not shown). Thus, multifaceted biological functions of AKR1B10 stimulated by arecoline may over-produce Ras protein and provoke oncogenesis.

OA, a natural compound distributed in various plants such as panax japonicus and clove, has low toxicity and modulates some biological roles to enhance hepatic functional protection, normal lipolytic metabolism, antiviral properties, and immune regulation. Notably, OA is reported to shorten the recovery time in patients with chemotherapy-related immunological dysfunction.[56,57] In addition, a recent study explored the efficacy of a combined nano-technique for forming CDDP/OA particles, reinforcing the pharmacokinetics intracellularly in apoptosis and rescuing multidrug resistance in CDDP therapy by using A549/CDDP cells and a xenograft tumor model in lung cancer.[58] OA has also been found to be the most potent and selective inhibitor of AKR1B10.[59] Mechanistically, OA suppresses multi-drug resistance proteins via activation of ROS/apoptosis signal-regulating kinase 1 (ASK1)/p38 MAPK pathway[60] and our data demonstrated that OA could significantly reduce CDDP resistance in a dose-dependent manner, as well as reduce stemness in OSCC cell lines by blockading AKR1B10 expression.

However, some limitations of the study should be noted. First, only 2 OSCC cell lines were used, affecting reproducibility. Second, an in vivo experiment in a xenograft tumor model is necessary to reproduce the results. Additionally, further studies are required to clarify how AKR1B10 promotes EMT-TFs such as Snail, Slug, and Twist to increase CSCs. Finally, it is also necessary to perform immunohistochemical studies on human oral samples to validate the findings.

In conclusion, we identified overexpressed AKR1B10 from SASsp cells and demonstrated that arecoline induced ROS/RNS to hyper-activate AKR1B10 production via the TGF-β1-Smad3 pathway. Furthermore, AKR1B10 enhanced CDDP-resistant OSCC cell lines via EMT-TFs and changing TMEs. Finally, Finally, OA may efficiently target CDDP resistance, reverse stemness in OSCC cells, and have the potential as a novel anticancer drug.

Declaration of competing interest

The authors have stated that there are no conflicts of interest related to this study.

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