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Secreted amphiregulin promotes vincristine resistance in oral squamous cell carcinoma

MING-JU HSIEH[1-4*], YIN-HONG CHEN[5*], I-NENG LEE[6], CHENG HUANG[7,8], YU-JU KU[9] and JUI-CHIEH CHEN[10]

[1]Oral Cancer Research Center, Changhua Christian Hospital, Changhua 500; [2]Institute of Medicine, Chung Shan Medical University, Taichung 402; [3]Graduate Institute of Biomedical Sciences, China Medical University, Taichung 404; [4]Department of Holistic Wellness, Mingdao University, Changhua 52345; [5]Department of Otorhinolaryngology-Head and Neck Surgery, Changhua Christian Hospital, Changhua 500; [6]Department of Medical Research, Chang Gung Memorial Hospital, Chiayi 61363; [7]Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei 112; [8]Department of Earth and Life Sciences, University of Taipei, Taipei 100; [9]The Center for General Education of China Medical University, China Medical University, Taichung 404; [10]Department of Biochemical Science and Technology, National Chiayi University, Chiayi 60004, Taiwan, R.O.C.

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Abstract. Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer. Despite advances in surgery, radiotherapy and chemotherapy, the overall 5-year survival rate of patients with OSCC has not significantly improved. In addition, the prognosis of patients with advanced-stage OSCC remains poor. Therefore, it is necessary to develop novel therapeutic modalities. Vincristine (VCR), a naturally occurring *vinca* alkaloid, is a classical microtubule-destabilizing agent and is widely used in the treatment of a number of cancers. Despite the proven antitumor benefits of VCR treatment, one of the major reasons for the failure of treatment is drug resistance. Changes in the tumor microenvironment are responsible for cross-talk between cells, which may facilitate drug resistance in cancers; secreted proteins may promote communication between cancer cells to induce the development of resistance. To identify the secreted proteins involved in VCR resistance, conditioned media was obtained, and an antibody array was conducted to screen a comprehensive secretion profile between VCR-resistant (SAS-VCR) and parental (SAS) OSCC cell lines. The results showed that amphiregulin (AREG) was highly expressed and secreted in SAS-VCR cells. Pretreatment with exogenous recombinant AREG markedly increased drug resistance against VCR in OSCC cells, as assessed by an MTT assay. Colony formation, MTT and western blot assays were performed to investigate the effects of AREG knockdown on VCR sensitivity. The results indicated that AREG expression can regulate VCR resistance in OSCC cells; overexpression of AREG increased VCR resistance in parental cells, whereas AREG knockdown decreased the VCR resistance of resistant cells. In addition, it was also demonstrated that the glycogen synthase kinase-3β pathway may be involved in AREG-induced VCR resistance. These findings may provide rationale to combine VCR with blockade of AREG-related pathways for the effective treatment of OSCC.

Correspondence to: Professor Jui-Chieh Chen, Department of Biochemical Science and Technology, National Chiayi University, 300 Syuefu Road, Chiayi 60004, Taiwan, R.O.C.
E-mail: jcc@mail.ncyu.edu.tw

*Contributed equally

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Introduction

Oral cancer refers any cancerous cells that are located in the oral cavity. It is a type of head and neck cancer, accounting for most head and neck cancers and leading to >145,400 cases/year of mortality globally (1). Oral squamous cell carcinoma (OSCC) is the most common type of malignancy in the oral cavity (2). Conventional treatment of OSCC includes surgery, radiotherapy and chemotherapy (3). Although the clinical outcome of patients with OSCC has gradually improved in the last few years, the prognosis of patients with advanced-stage disease remains poor, reflecting limited advances in present understanding of the pathogenesis of this disorder (4). Therefore, it is necessary to develop novel therapeutic approaches for patients with advanced and unresectable OSCC.

Vincristine (VCR), a naturally occurring *Vinca* alkaloid, is a classical microtubule-destabilizing agent (5). It is widely used for hematologic malignancies and certain solid tumors (6-8). However, despite the proven antitumor activity of VCR treatment, the efficacy of chemotherapy is often limited by the rapid emergence of acquired resistance and patient relapse following initial response.

Tumors consist of a complex microenvironment composed of cancer cells, stroma and immune cells (9). Drug resistance is a complex process involving reciprocal interplay between different types of cells. Secreted proteins are responsible for the cross-talk among cells, which may facilitate drug resistance in tumors (10-12).

Several studies have indicated that soluble mediators from the microenvironment can promote cancer growth and therapy resistance (13-15). Amphiregulin (AREG) (16), a ligand of the epidermal growth factor (EGF) receptor (EGFR), is synthesized as a transmembrane precursor that undergoes a series of proteolytic steps to produce mature forms for secretion (16). AREG has been reported to induce oncogenic effects in numerous cancer cell types, including breast, liver, pancreatic and colorectal cancer cells (17-20) and to be implicated in drug resistance (10,18,21,22); however, the effects of AREG and its mechanisms of action in OSCC cells remain unknown. Understanding the complex mechanisms underlying its effects may reveal potential therapeutic opportunities.

Antibody arrays possess valuable applications in cancer research to identify biomarkers or molecules that are potentially relevant for diagnosis, prognosis, treatment and drug development (23). To elucidate the association between secreted proteins and VCR resistance in OSCC cells, a VCR-resistant SAS subline (SAS-VCR) was established by exposure to an increasing drug concentration gradient. Conditioned medium (CM) was collected from parental and VCR-resistant cells, and the secreted proteins were assessed using an antibody array. In the present study, comprehensive secretion profiling was performed to provide novel insight in the mechanisms of VCR resistance. Understanding the relationship between secreted proteins and drug resistance may contribute to the development of novel therapeutic strategies and biomarkers in OSCC.

Materials and methods

Chemicals, reagents and antibodies. VCR, 5-fluorouracil (5-FU), cisplatin, MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Merck KGaA). VCR was dissolved in sterile PBS and diluted in cell culture medium to the required concentration prior to use. 5-FU was dissolved in DMSO. Cisplatin was dissolved in dimethylformamide. A potent glycogen synthase kinase-3 (GSK-3) inhibitor, LY2090314 (Selleck Chemicals), was dissolved in DMSO. The antibodies used in this study were as follows: Cleaved poly (ADP-ribose) polymerase (1:1,000; PARP; cat. no. 9541) was purchased from Cell Signaling Technology, Inc.; AREG (1:200; cat. no. sc-74501), Bcl-2 (1:200; cat. no. sc-7382), phosphorylated (p)-GSK-3β (1:200; cat. no. sc-135653) and GSK-3β (1:200; cat. no. sc-9166) were purchased from Santa Cruz Biotechnology, Inc.; α-tubulin (1:10,000; cat. no. 05-829) was purchased from EMD Millipore. Human recombinant AREG (rAREG) was purchased from R&D Systems, Inc.

Cell culture. The human OSCC cell lines, SAS and SCC9, were kindly provided by Dr Ming-Chang Hsieh (Chung Shan Medical University Hospital, Taichung, Taiwan). All cells were cultured in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 2 mM glutamine, and maintained at 37˚C in a humidified atmosphere of 5% CO_2. To investigate the mechanism of VCR resistance in OSCC, SAS-VCR and SCC9-VCR cells were established over ~6 months by gradually increasing the concentration of VCR in the culture medium by 0.5 to 16 nM and 0.125 to 4 nM, respectively.

Cell viability assay and treatments. An MTT assay was conducted to evaluate the effects of VCR on the viability of OSCC cells. Briefly, cells were seeded into the wells of 96-well plates at a density of 5,000 cells in 100 μl of culture medium. Following overnight incubation to allow the attachment of cells, cells were incubated with 0-64 nM VCR in serum-free medium at 37˚C. At 0-48 h time intervals following VCR treatment, 30 μl of MTT (5 mg/ml) was added to each well and incubated for a further 4 h at 37˚C. The supernatant was then discarded, and 100 μl DMSO was added to each well to dissolve the formazan crystals. The optical density was evaluated by measuring the absorbance, with a test wavelength of 490 nm and a reference wavelength of 630 nm.

To investigate whether CM can enhance VCR resistance, CM was collected as described in the 'Collection of CM' section. SAS cells were activated by CM from SAS and SAS-VCR cells for 8 h, and then treated with 8 μM VCR for 24 h.

To further determine the relationship between AREG and VCR resistance, SAS cells were pretreated with SAS-VCR-CM in combination with or without AREG-neutralizing antibody (1:500) for 8 h, and then treated with 8 μM VCR for 24 h.

For the effect of rAREG on VCR sensitivity, SAS cells were pretreated with rAREG (50 ng/ml) for 4 h and then stimulated with 16 μM VCR for 48 h. SCC9 cells were pretreated with rAREG (100 ng/ml) for 4 h and then stimulated with 4 μM VCR for 24.

To analyze whether serum starvation can induce expression of AREG, $5x10^5$ cells/well were seeded in 6-well plates and cultured overnight. Following attachment, cells were washed twice with PBS, and then serum-free DMEM/F12 was added. Cells were analyzed by MTT and western blot assays after 12, 24 and 48 h.

To investigate whether inhibition of GSK3-β activation can block AREG-induced VCR resistance, cells were pretreated with LY2090314 (20 nM) for 30 min, followed by treatment with rAREG (50 ng/ml) for 4 h and then treatment with 4 μM VCR for 24 h. Cell viability was assessed by an MTT assay.

To investigate the sensitivity of SAS and SAS-VCR cells to 5-FU and cisplatin, cells were treated with 0-160 μM 5-FU for 48 h or 0-20 μM cisplatin for 24 h, and then analyzed using an MTT assay.

Colony formation assay. Cells were cultured a 6-well plate at a density of $5x10^4$ cells/well with regular medium. Cells were treated with the 0-64 nM VCR concentrations for 24 h. Then, the cells were seeded at $1x10^5$ cells/well into a separate 6-well plate. Cells were allowed to grow until colonies were visible (5-6 days) and then fixed with methanol for 30 min and stained with 0.5% crystal violet for 30 min at room temperature (RT). Images of the colonies were acquired using a digital camera. Colonies were counted using ImageJ 1.52a software (National Institutes of Health).

Western blot analysis. Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). The protein concentration was determined using a Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Proteins from the total cell lysates or CM (40 μg/lane) were separated by via 8-15% SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were blocked with 5% skim milk for 1 h at RT and then probed with the indicated primary antibodies for 1 h at RT. Following three washes with TBS-0.1% Tween 20, the membranes were incubated with the HRP-conjugated anti-mouse (cat. no. 20102) or anti-rabbit IgG antibody (cat. no. 20202; both 1:5,000; Leadgene Biomedical, Inc.) for 1 h at RT. The blots were visualized using ECL reagent (PerkinElmer, Inc.) and autoradiography.

Collection of CM. Cells seeded in 10-cm dishes were grown to 80% confluence and then washed with PBS twice. Cells were subsequently incubated in serum-free media for 48 h. CM was collected via gentle aspiration and then centrifuged at 875 x g at RT for 10 min to remove cell debris. The CM was further concentrated using Amicon® Ultra 15 ml centrifugal filters with a 3-kDa cut-off (EMD Millipore) at 4,000 x g and 4˚C to a total volume of ~150-200 ul. The CM was aliquoted and stored at -20˚C prior to use.

Growth factor human antibody array. A human growth factor antibody array (cat. no. ab134002; Abcam) was used according to the manufacturer's protocols; all reagents listed below were included in this array unless otherwise specified. Briefly, the concentrated CM (200 μg total protein) was mixed with blocking buffer and incubated with membranes at 4˚C overnight. Membranes were then washed and incubated with biotin-conjugated anti-cytokines for 2 h at RT, followed by washing and incubation with HRP-conjugated streptavidin for 2 h at RT. Membranes were then washed again, and bound antibodies were visualized using ECL reagents and autoradiography. The relative expression was determined using UN-SCAN-IT gel 6.1 software (Silk Scientific, Inc.). The average signal of a pair of duplicate spots was normalized using negative control spots as a background value. The relative intensities in SAS-VCR cells were determined by comparing the corresponding signals to SAS cells.

Oncomine database. The Oncomine Cancer Microarray database (http://www.oncomine.org/) was used to study the expression levels of AREG in human oral tumor and normal tissues obtained from separate individuals. The gene expression data were log transformed, median centered per array, and the standard deviation was normalized to 1 per array. A gene was considered as overexpressed when its mean value in tumor samples was significantly increased compared with its mean value in normal tissue as determined using a t-test ($P \leq 0.05$) and the fold change was ≥1.5.

Lentivirus infection and short hairpin (sh)RNA knockdown. The pLKO.1-puro-based lentiviral plasmids containing TRCN0000117995-shAREG (sequence: 5'-CCGGGAACG AAAGAAACTTCGACAACTCGAGTTGTCGAAGTTTCT TTCGTTCTTTTTG-3')andpLKO.1-shScramble(sequence:5'-CCG GCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCG ACTTAACCTTAGGTTTTT-3') were obtained from National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). All plasmids (4 μg lentiviral plasmid; 4 μg pCMVΔR8.91; 0.4 μg pMD; all Academia Sinica) were cotransfected into 293T cells ($5x10^5$; cat. no. 632180; Clontech Laboratories, Inc.) in 6-cm dishes using TurboFect (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The lentivirus-containing supernatants were harvested at 48 h post-transfection. SAS-VCR cells ($1x10^5$) were infected using the lentivirus-containing supernatant (12,762 RIU/μl). For stable cell lines, the infected cells were selected by puromycin (5 μg/ml) within 1 week.

AREG overexpression. An AREG overexpression plasmid (pCMV3-AREG) and negative control (pCMV3) were purchased from Sino Biological Inc. Plasmids were transfected into the two OSCC cell lines using TurboFect according to the manufacturer's protocol. Cells ($1x10^5$) were seeded in each well of a 24-well plate. After culturing for 24 h, cells were transiently transfected under optimized transfection conditions. Briefly, 1 μg of DNA plasmid DNA was diluted in 100 μl of serum-free DMEM/F12, and mixed with 2 μl of transfection reagent followed by incubation at RT for 20 min. The mixture was then added dropwise to the cells and incubated for an additional 72 h at 37˚C in a humidified atmosphere and 5% CO_2.

Statistical analysis. All values represent the mean ± SEM from at least 3 independent experiments. Student's t-test was used when comparing two independent groups. Statistical comparisons of >2 groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test. In all cases, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment and characterization of VCR-resistant SAS cells. As presented in Fig. 1A, SAS-VCR cells (IC_{50} >1,024 nM) were more resistant to VCR than their respective parental SAS cells (IC_{50}=63.96±0.25 nM). In addition, a colony formation assay was also conducted. As presented in Fig. 1B, the colony numbers of SAS cells were significantly decreased compared with control treatment in dose-dependent manner; however, the SAS-VCR colony number was significantly decreased compared with the control only in the high dose group (64 nM). Next, the effects of VCR on the expression of cleaved PARP, a marker of apoptosis, and Bcl-2 (an antiapoptotic protein) were evaluated via western blotting. As shown in Fig. 1C, the expression of cleaved PARP was notably induced in VCR-treated SAS cells. In contrast, the expression of cleaved PARP was not observed in SAS-VCR cells following exposure to 8 or 16 nM VCR for 24 h, indicating that SAS-VCR cells were highly resistant to VCR compared with SAS cells. The expression of Bcl-2 was increased in a dose-dependent manner in VCR-treated SAS-VCR cells, whereas the expression of Bcl-2 was decreased in a dose-dependent manner in VCR-treated SAS cells. These results indicated that SAS-VCR cells were resistant to VCR compared with SAS cells. Furthermore, the

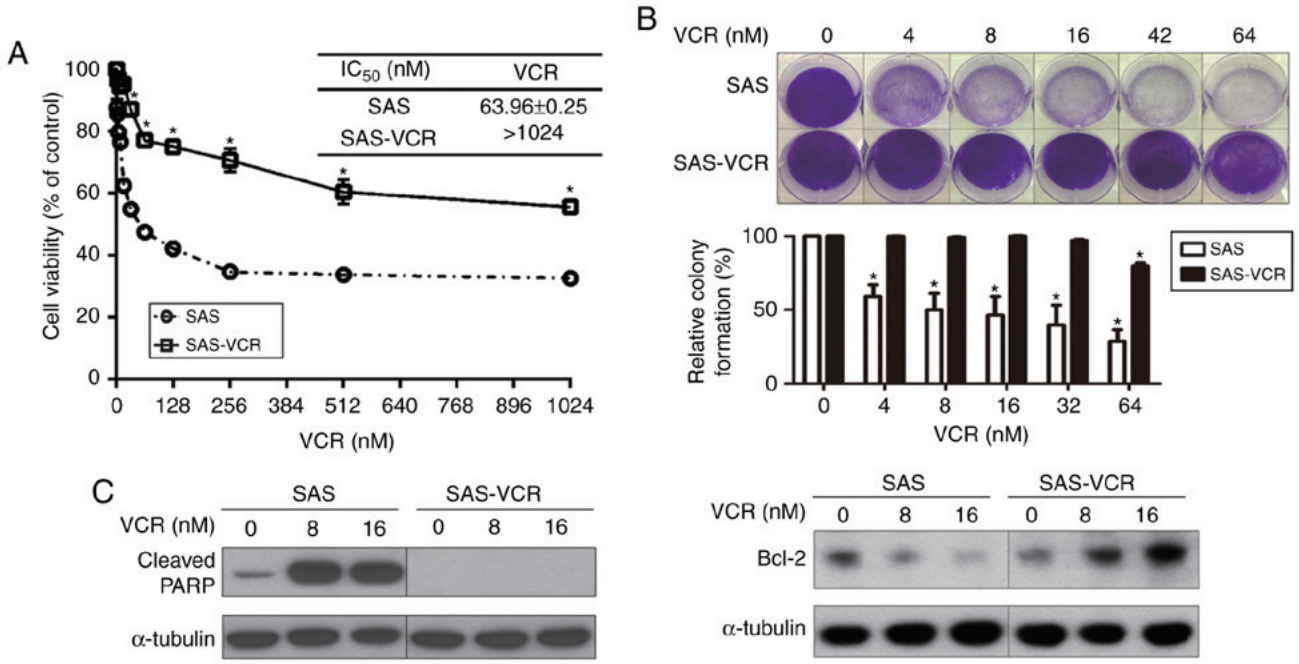

Figure 1. Cytotoxic effects of VCR on SAS and SAS-VCR cells. (A) Cells were treated with various concentrations of VCR for 48 h and cell viability was measured by an MTT assay. Data are presented as the mean ± SEM of four independent experiments. *P<0.05 vs. SAS. (B) Long-term effects of VCR were assessed using a colony formation assay. Above, representative images showing that SAS cells formed fewer colonies compared with SAS-VCR cells. Below, densitometric analysis of the clonogenic growth of SAS and SAS-VCR cells. Data are presented as the mean ± SEM of three independent experiments. *P<0.05 vs. 0 μM of the respective cell type. (C) Cells were treated with VCR (0, 8 or 16 μM) for 24 h, and the expression levels of apoptosis-associated proteins (cleaved PARP and Bcl-2) were examined via western blotting. α-tubulin was used as a loading control. PARP, poly (ADP-ribose) polymerase; SAS-VCR, VCR-resistant SAS cells; VCR, vincristine.

sensitivity of the SAS-VCR cell line to 5-FU and cisplatin was also explored, and it was revealed that only resistance to 5-FU was observed in these cells, suggesting a potential link between resistance to VCR and 5-FU resistance (Fig. S1).

Comparison of growth factor profiles in the secretomes of SAS and SAS-VCR cells. To evaluate whether the secreted proteins from resistant cells were associated with the induction of drug resistance, CM was obtained from SAS and SAS-VCR cells. Notably, the CM from SAS-VCR cells significantly increased VCR resistance when applied to parental SAS cells (Fig. 2A). Subsequently, antibody arrays were used to analyze the differences in the secretomes of CM from SAS and SAS-VCR cell lines. As presented in Fig. 2B, a total of 22 secreted proteins were identified whose expression was changed >1.5-fold between the two media. The levels of 17 secreted proteins were increased, and those of 5 secreted proteins was decreased in SAS-VCR. Of these, the levels of AREG, basic fibroblast growth factor, heparin-binding EGF-like growth factor, platelet-derived growth factor-AB, placental growth factor and vascular endothelial growth factor D secretion were most notably upregulated (>4-fold) in SAS-VCR CM, indicating that the secreted proteins may be important mediators of VCR resistance in OSCC cells.

AREG is highly expressed and secreted to promote VCR resistance in OSCC cells. To determine the clinical relevance of these secreted proteins, the Oncomine database was employed to select the appropriate target for further study. The results revealed that there were only clinical data concerning the expression of AREG; its expression was significantly upregulated in carcinoma tissue compared normal oral cavity tissue (Fig. 2C). In addition, among the six proteins, numerous studies indicated that AREG serves a critical role in OSCC (24-26); however, its role in VCR sensitivity is yet to be described in the literature. To further confirm the antibody array results, western blotting was performed to determine if AREG was highly expressed and secreted in SAS-VCR cells. As shown in Fig. 2D, the expression of AREG in the cell lysates was analyzed, and the results indicated that expression of AREG was increased in SAS-VCR cells compared with in SAS cells. In addition, the levels of AREG in CM were also significantly elevated for SAS-VCR cells compared with SAS cells (Fig. 2E). To provide further evidence that AREG mediates VCR sensitivity, AREG activity was blocked using a neutralizing antibody. The results showed that pretreatment of CM with neutralizing antibodies against AREG restored VCR sensitivity in SAS cells (Fig. 2F).

AREG also modulates VCR sensitivity in SCC9 cells. To further validate the role of AREG in VCR sensitivity, SAS cells were pretreated with rAREG and then stimulated with VCR. The results showed that AREG markedly increased resistance to VCR in SAS cells (Fig. 3A). Similarly, rAREG also enhanced VCR resistance in SCC9 cells (Fig. 3B). Furthermore, the expression levels of AREG and VCR resistance in the two cell lines were measured. The results showed that the expression of AREG was higher in the more VCR-resistant cell line, SAS (Fig. 3C). To clarify whether AREG expression is associated with VCR

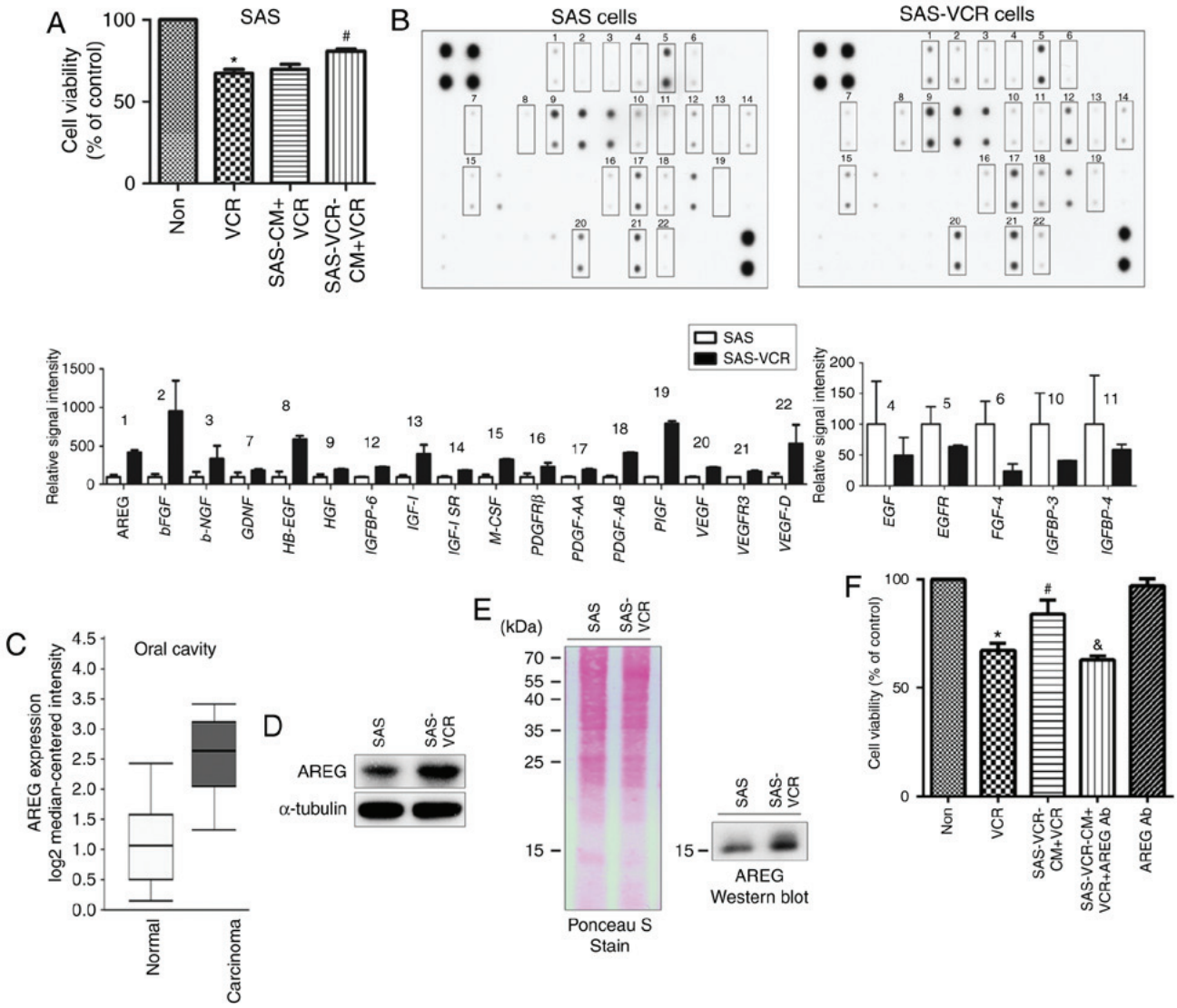

Figure 2. Growth factors secreted by SAS-VCR cells may promote resistance to VCR. (A) Secreted substances of SAS-VCR cells can promote VCR resistance in SAS cells. Data are presented as the mean ± SEM of three independent experiments. *P<0.05 vs. Non; #P<0.05 vs. VCR. (B) Comparison of growth factors in CM from SAS and SAS-VCR cells was performed using a human growth factor antibody array. The expression of the marked molecules was altered. Below, densitometric analysis of the pair of duplicate spots representing each marked protein. (C) Box plots derived from gene expression data from the Oncomine cancer database comparing expression of AREG gene in normal and carcinoma tissue. The fold change is 2.668 and P-value is $6.15x10^{-9}$. (D) Analysis of AREG levels in total cell lysate of SAS and SAS-VCR as determined via western blotting. α-Tubulin was used as a loading control. (E) Equal volumes of CM from SAS and SAS-VCR cells was analyzed for secreted AREG via western blotting. Loading quantities were shown on left side by Ponceau S staining. (F) Cells were pretreated with SAS-VCR-CM and/or AREG-neutralizing antibody, and then treated with 8 μM VCR for 24 h. Cell viability was determined by an MTT assay. Data are presented as the mean ± SEM of at least four independent experiments. *P<0.05 vs. Non; #P<0.05 vs. VCR; &P<0.05 vs. SAS-VCR-CM + VCR. Ab, antibody; AREG, amphiregulin; CM, condition medium; Non, untreated cells; SAS-VCR, VCR-resistant SAS cells; VCR, vincristine.

sensitivity in SCC9 cells, a VCR-resistant SCC9 subline termed SCC9-VCR was established. According to the results of the MTT assay, SCC9-VCR cells were significantly more viable following VCR treatment compared with SCC9 cells, as assessed at 24 and 48 h (Fig. 3D and E). Furthermore, SCC9 cells exhibited increased expression of cleaved PARP compared with SCC9-VCR cells following VCR treatment (Fig. 3F). To further evaluate the association between AREG levels and VCR resistance, SCC9 and SCC9-VCR cells were treated with increasing doses of VCR, and then the expression of AREG was analyzed via western blotting. The results revealed that SCC9-VCR cells exhibited upregulated AREG expression compared with SCC9 cells. Furthermore, an increase in the expression levels of AREG was observed in SCC9-VCR cells, but not SCC9 cells, after treatment with increasing doses of VCR (Fig. 3G).

Knockdown of AREG restores VCR sensitivity, and overexpression of AREG confers resistance to VCR. To determine the impact of AREG expression on VCR sensitivity, AREG expression was suppressed in SAS-VCR cells using a lentivirus-mediated RNA interference system. The knockdown efficiency of shAREG was evaluated via western blotting (Fig. 4A). Silencing AREG expression resulted in elevated sensitivity to VCR in a dose-dependent manner compared with shControl cells, as determined by MTT (Fig. 4B) and colony formation assays (Fig. 4C). Furthermore, AREG knockdown notably promoted cell apoptosis in SAS-VCR cells, as

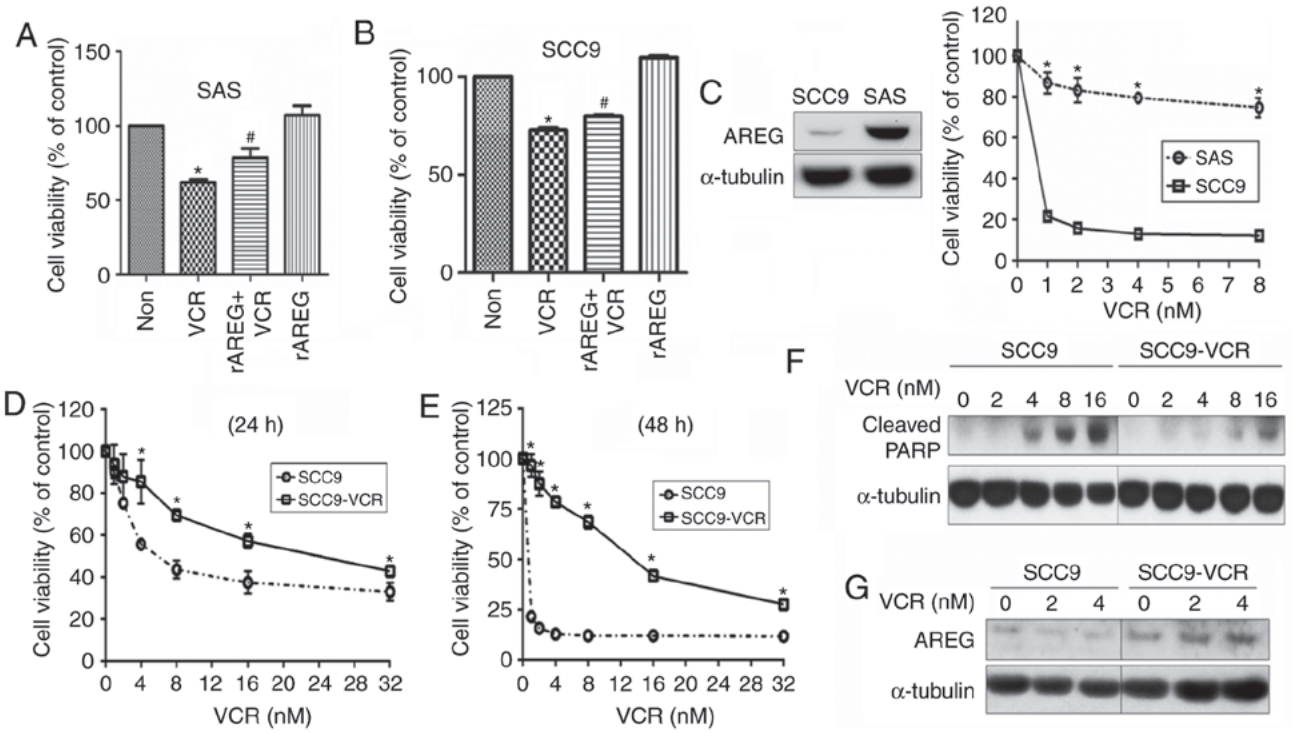

Figure 3. AREG is involved in VCR resistance in oral squamous cell carcinoma cells. Pretreatment of rAREG confers VCR resistance in (A) SAS and (B) SCC9 cells. SAS cells were pretreated with rAREG (50 ng/ml) for 4 h and then stimulated with 16 μM VCR for 48 h (n=6). SCC9 cells were pretreated with rAREG (100 ng/ml) for 4 h and then stimulated with 4 μM VCR for 24 h (n=3). Cell viability was examined by an MTT assay. Data are presented as the mean ± SEM. *P<0.05 vs. Non; #P<0.05 vs. VCR. (C) Left, AREG levels in cell lysates from SCC9 and SAS cells were analyzed via western blotting. Right, SCC9 and SAS cells were exposed to indicated doses of VCR for 24 h and assayed for survival by an MTT assay. Data are presented as the mean ± SEM of at least four independent experiments. *P<0.05 vs. SCC9. Comparison of VCR sensitivity between parental SCC9 and SCC9-VCR cells at (D) 24 and (E) 48 h as determined by an MTT assay (n=4). *P<0.05 vs. SCC9. (F) SCC9 and SCC9-VCR cells were treated with various concentrations of VCR (0-16 μM), for 24 h and the expression levels of cleaved PARP were examined via western blotting. (G) Dose-dependent effects of VCR on the expression of AREG in SCC9 and SCC9-VCR cells were analyzed via western blotting. α-tubulin was used as a loading control. AREG, amphiregulin; Non, untreated cells; PARP, poly (ADP-ribose) polymerase; r, recombinant; SAS-VCR/SCC9-VAR, VCR-resistant SAS/SCC9 cells; VCR, vincristine.

evidenced by an increase in the cleavage of PARP at a concentration of 64 nM VCR for 24 h (Fig. 4D). The results indicated that the knockdown of AREG may increase VCR-induced apoptosis in OSCC cells. To verify the relevance of AREG in mediating resistance to VCR, cells were then transiently transfected with AREG expression vector or control vector. AREG was overexpressed in SAS and SCC9 cells following transfection, as determined via western blotting (Fig. 4E). As presented in Fig. 4F, overexpression of AREG in SAS and SCC9 cells significantly increased resistance to VCR, as assessed using an MTT assay. Furthermore, whether AREG overexpression protected against starvation-induced death in OSCC cells was analyzed. The result revealed that overexpression of AREG significantly increased the viability of serum-starved SAS cells at 48 h compared with cells transfected with a control vector (Fig. 4G). Furthermore, whether serum starvation can affect the expression of AREG, which may prevent cell death and promote resistance to harsh environments such as drug treatment, was evaluated. As presented in Fig. 4H, serum deprivation notably induced AREG expression in SAS and SCC9 cells in a time-dependent manner. These results indicated that AREG was involved in protecting OSCC cells against various stresses, including VCR treatment.

AREG regulates VCR sensitivity in OSCC cells via activation of GSK-3β. A previously study reported that AREG can modulate the GSK-3β pathway to regulate cell functions; GSK-3β is known to be a potential therapeutic target for cancer treatment (27). In addition, it has been demonstrated that targeting the GSK-3β pathway may be beneficial for the treatment of oral cancer (28). Therefore, the activation of GSK-3β was analyzed after treatment of SAS and SAS-VCR cells with increasing concentrations of VCR. The results showed that VCR induced a dose-dependent decrease in GSK-3β phosphorylation in SAS cells, whereas GSK-3β maintained sustained activation in SAS-VCR cells (Fig. 5A). In addition, a marked downregulation of p-GSK-3β was also observed in VCR-treated SAS-VCR/shAREG cells compared with SAS-VCR/shControl cells (Fig. 5B). To further confirm that AREG can indeed activate the GSK-3β pathway, GSK-3β phosphorylation was directly analyzed in response to rAREG. The results revealed that treatment of SAS cells with rAREG can induce an increase in the phosphorylation of GSK-3β in a time-dependent manner (Fig. 5C). Then, a GSK-3β inhibitor, LY2090314, was used to interfere with AREG-induced GSK-3β activation and observe whether blocking GSK-3β activation would affect the role of AREG in VCR resistance. It was demonstrated that rAREG induced a significant increase in VCR resistance; however, this effect was significantly attenuated by LY2090314 (Fig. 5D). These findings suggested that AREG can regulate the activation of GSK-3 to promote VCR resistance in OSCC cells.

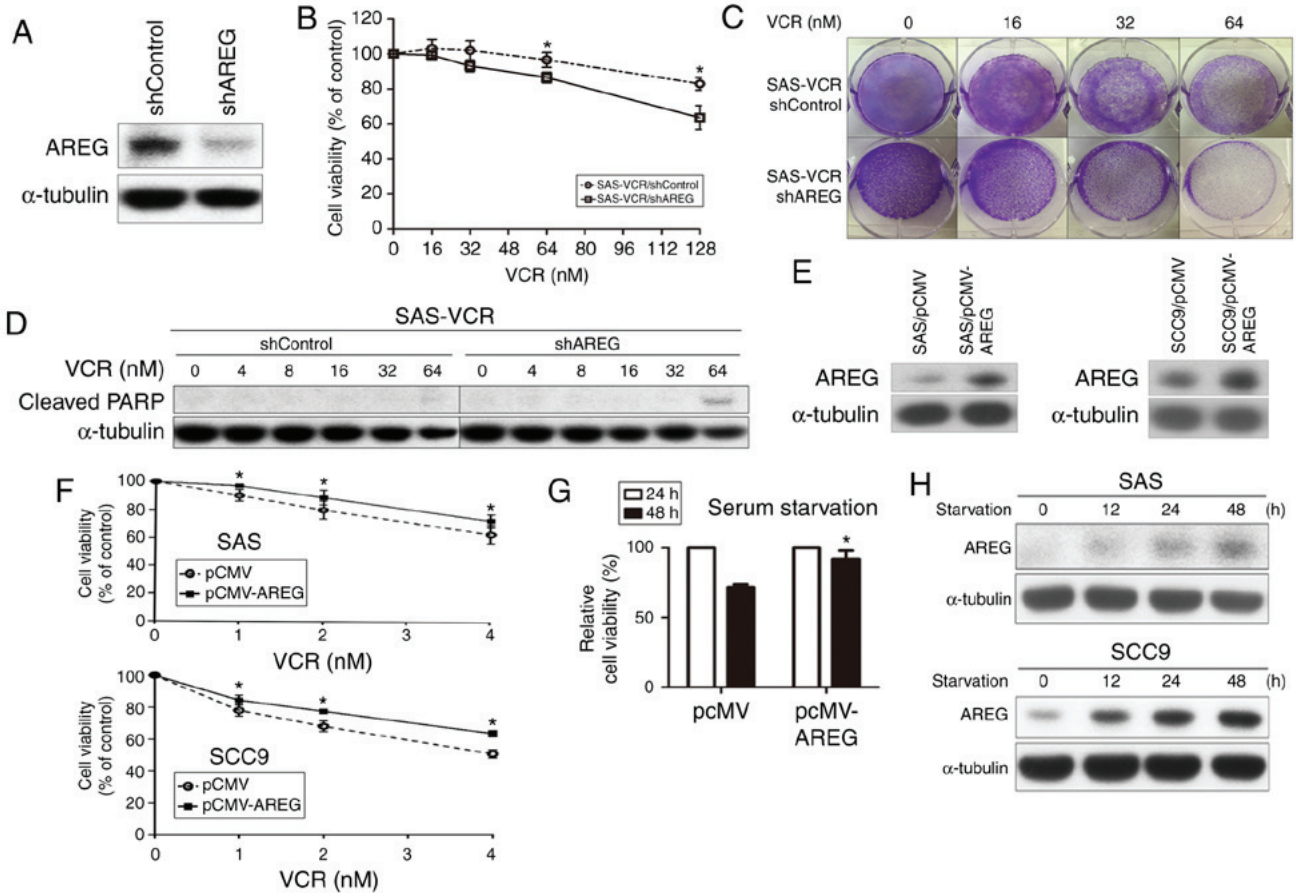

Figure 4. Effects of knockdown or overexpression of AREG on VCR sensitivity in oral squamous cell carcinoma cells. (A) Efficiency of AREG knockdown was verified via western blotting. (B) Cells were exposed to increasing concentrations of VCR for 48 h and subsequently evaluated by an MTT assay (n=4). *P<0.05 vs. SAS-VCR/shAREG. (C) SAS-VCR/shAREG and SAS-VCR/shControl were treated with the indicated concentrations of VCR and then subjected to colony formation assays. (D) SAS-VCR/shControl and SAS-VCR/shAREG cells were treated with increasing concentrations of VCR for 24 h, and cleaved PARP expression was analyzed via western blotting. (E) Expression of AREG was analyzed in whole cell lysates using western bloting. α-Tubulin was used as a loading control. (F) Overexpression of AREG induces VCR resistance in SAS and SCC9 cells. SAS cells were treated with the indicated concentrations of VCR for 48 h. SCC9 cells were treated with the indicated concentrations of VCR for 24 h. Data are presented as the mean ± SEM of four independent experiments. *P<0.05 vs. pCMV. (G) MTT assay to determine the effects of AREG overexpression on the viability of serum-starved SAS cells (n=4). *P<0.05 vs. pCMV at 48 h. (H) Effects of serum starvation on AREG expression in OSCC cells. Cells were cultured in serum-free medium for various durations and then analyzed via western blotting. AREG, amphiregulin; PARP, poly (ADP-ribose) polymerase; SAS-VCR, VCR-resistant SAS cells; sh, short hairpin (RNA); VCR, vincristine.

Discussion

A substantial body of evidence has revealed that upregulated expression of AREG is associated with cancer progression in a wide variety of cancers, including lung (29,30), breast (17,31), ovarian (32,33), liver (18,34), pancreatic (19,35) and colorectal cancers (20,36). At present, however, there has been no study into the function of ARGE in relation to drug resistance in OSCC. In the present study, an antibody array was performed to explore the secretion profile of OSCC cells, and it was revealed that AREG was highly expressed and secreted in VCR-resistant cells. In addition, it was also suggested that the GSK-3β pathway may be involved in AREG-induced VCR resistance. These findings may aid the development of novel therapeutic targets for OSCC treatment and improved prognosis.

Chemotherapy is widely used in the treatment of OSCC, and VCR is a classic microtubule-destabilizing agent that is effective and widely used in hematological malignancies and certain solid tumors (37). As VCR exhibits substantial anticancer activity, it may be a potential treatment for OSCC. However, several studies have indicated that high-dose VCR is associated with a significant risk of severe gastrointestinal toxicity; mortality from treatment-related toxicity has been previously reported in patients (38,39). Therefore, combining VCR with other agents or molecules is may improve the clinical management of oral cancers. Certain drugs with antitumor activity have been reported to increase the VCR sensitization of VCR-resistant oral epidermoid carcinoma cells (40-42). Though great efforts have been made in developing novel anticancer drugs with increased curative potential or the ability to reverse drug resistance, the results remain satisfactory, due to either a lack of potency or unacceptable side effects.

Proteins secreted, shed or leaking from cancer cells, collectively termed the cancer secretome, are considered promising biomarkers, as they may be detectable in blood or other body fluids (43-45). Previous studies indicated that cancer cells can secrete soluble mediators in response to drug therapy, which contribute to the promotion of drug resistance and tumor progression (46,47). In addition, secreted proteins

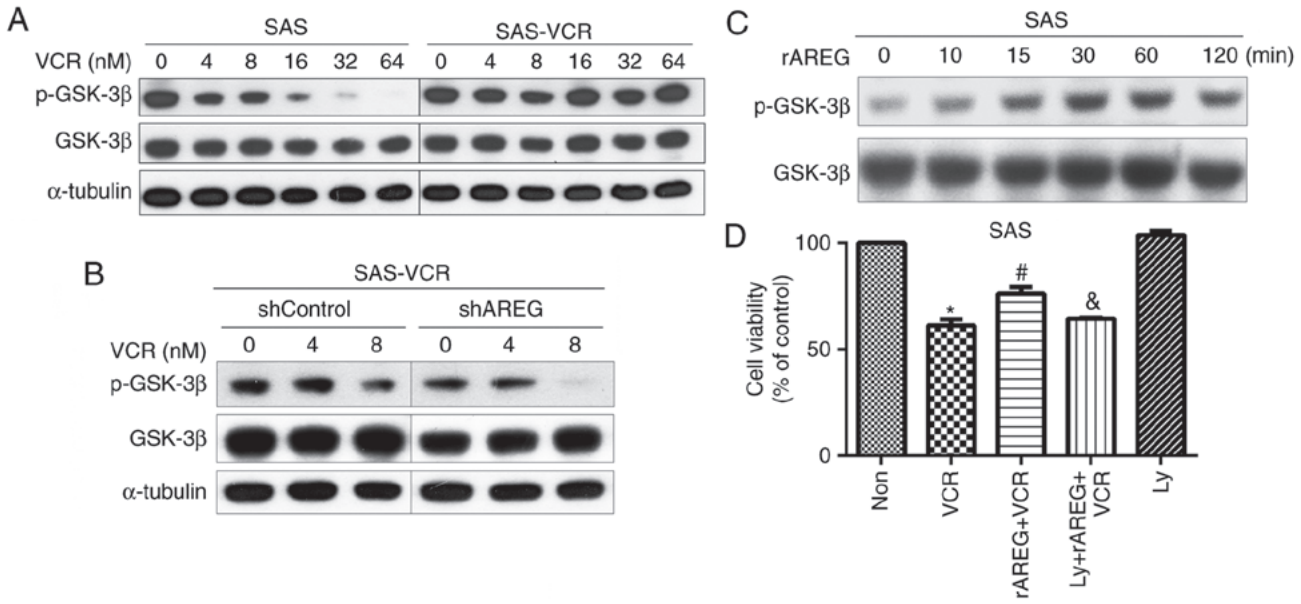

Figure 5. GSK3-β signaling pathways may be involved in the effects of AREG on VCR sensitivity. (A) SAS and SAS-VCR cells were treated with different concentrations of VCR (0-64 nM) for 24 h. (B) SAS-VCR/shControl and SAS-VCR/shAREG cells were exposed to various concentrations of VCR for 24 h. (C) SAS cells were incubated with rAREG (50 ng/ml) for the indicated time intervals. p-GSK3-β and GSK3-β were analyzed via western blotting. (D) Cells were pretreated with Ly (20 nM) for 30 min, followed by treatment with rAREG (50 ng/ml) for 4 h, and then treatment with 4 μM VCR for 24 h. Cell viability was evaluated by an MTT assay. Data are presented as the mean ± SEM (n=5). *P<0.05 vs. Non; $^{\#}$P<0.05 vs. VCR; $^{\&}$P<0.05 vs. rAREG + VCR. AREG, amphiregulin; GSK-3β, glycogen synthase kinase-3β; Ly, LY2090314; Non, untreated cells; p, phosphorylated; r, recombinant; SAS-VCR, VCR-resistant SAS cells; VCR, vincristine.

are also considered good candidate serological tumor markers, as they are released by the cells and thus exhibit the greatest possibility of entering the circulation (48). Therefore, secreted proteins are regarded as a rich source of potential markers and drug targets for cancer treatment (49).

AREG is an EGF-like ligand that has been identified as a ligand responsible for EGFR-ERK signaling activation, which can lead to cancer progression (19,50-52). Regarding drug sensitivity, AREG is upregulated in non-responding patients compared with patients who do respond to gefitinib (21). In animal models, a previous study has shown that AREG silencing can reduce the size of tumor growth and increase drug sensitivity in glioma cells (53). As AREG is a secreted protein, it can enter the circulatory system. A separate study showed that circulating AREG could be clinically relevant as an indicator of unfavorable response to gefitinib in NSCLC (54). In Taiwan, oral cancer accounts for the fourth highest incidence of malignancy in males (55). Numerous studies indicate that EGFR is frequently overexpressed in human OSCC (56-59).

High EGFR expression has been associated with resistance to chemotherapeutic agents used in the treatment of OSCC, including cisplatin, 5-FU, cyclophosphamide and doxorubicin, suggesting that EGFR signaling may be a promising target for OSCC therapy (58-60). Cetuximab is a chimeric IgG1-human antibody targeted against the extracellular domain of EGFR (61). It was approved by the FDA in 2006 as a component of combination therapy along with radiation and/or chemotherapy to treat OSCC; however, clinical use of cetuximab is limited, as EGFR expression levels have not been associated with response levels to cetuximab (62,63). At present, in OSCC, clinically relevant mechanisms of cetuximab resistance have not been clearly elucidated.

GSK-3β, a multifunctional serine/threonine kinase, was originally discovered as a key regulator of glycogen metabolism (64). Accumulating evidence suggests that it involved in tumorigenesis, migration, invasion, chemotherapy and drug resistance (65,66). Therefore, GSK-3β has emerged as a potential therapeutic target for different types of cancers (67,68). Recent research in OSCC has shown that GSK-3β can regulate matrix metalloproteinase-9 activity to promote cancer progression and invasion (69). In clinical specimens, another study also showed that the levels of p-GSK-3β and GSK-3β in OSCC tissues are upregulated compared with in controls, and are positively associated with tumor metastasis and poor survival in patients (70). Although a number of studies have indicated that multidrug resistance can be reversed by inhibiting GSK-3β (71-73), whether this occurs in OSCC remains unclear. The relationship of GSK-3β with the drug treatment of OSCC remains to be further explored in future studies.

In conclusion, to improve understanding of the mechanisms underlying drug resistance in OSCC, a VCR-resistant OSCC cell line was established, and the secreted proteins were analyzed using an antibody microarray. This study is the first, to our knowledge, to characterize changes in the secretome of VCR-resistant OSCC cells. The results indicated that AREG was highly expressed and secreted in VCR-resistant cells compared with VCR-sensitive cells. Pretreatment with exogenous rAREG markedly increased drug resistance against VCR in OSCC cells. Furthermore, knockdown of AREG increased VCR sensitivity, whereas overexpression of AREG further promoted VCR resistance. The results indicated that AREG contributes to VCR resistance in OSCC cells. Additionally, it was also demonstrated that the GSK-3β pathway may be involved in AREG-induced VCR resistance. These findings

may provide valuable insight for the development of effective treatments against OSCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JCC was involved in the study design, data analysis and drafting the manuscript. MJH and YHC performed the experiments and data acquisition. INL, CH and YJK contributed to data analysis and interpretation.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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