Secretory Kin17 is Correlated with Chemoresistance in Oral Squamous Cell Carcinoma

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Abstract: *Purpose*: Kin17 is a conserved nuclear protein that participates in DNA damage repair, DNA replication and cell proliferation. Several reports have linked Kin17 to tumor progression. However, the role of Kin17 in oral squamous cell carcinoma (OSCC) has not yet been described. The aims of this study were to assess Kin17 transcript and protein expression in OSCC and to evaluate an association for this protein with chemoresistance.

Methods: Kin17 expression in OSCC tissues and OSCC cell lines was measured by standardized immunohistochemistry, western blotting and semi-quantitative RT-PCR. Secretory Kin17 protein was measured in serum samples and cell culture conditioned media. A recombinant Kin17 protein was purified and used in a chemoresistance assay.

Results: Kin17 was identified as an unconventional secretory protein, whose expression levels were correlated with chemotherapy and chemoresistance in OSCC. Kin17 protein expression was up-regulated in patients exhibiting chemoresistance. Serum Kin17 levels were significantly increased in patients receiving chemotherapy. We provide evidence that the secretory Kin17 protein plays a role in the DNA damage response in OSCC. Furthermore, we also show that the secretory Kin17 protein enhances the chemoresistance of OSCC cells and increases the expression of multidrug resistant genes.

Conclusion: To our knowledge, this is the first report of Kin17 being characterized as a secretory protein. This novel role for Kin17 may have implications for studying the chemoresistance process in OSCC. The effective inhibition of Kin17 secretion may improve or prolong chemotherapeutic effects, making it an attractive therapeutic target candidate for further study.

Keywords: Kin17, secretory protein, oral cancer, DNA damage, chemoresistance.

INTRODUCTION

Kin17, a conserved nuclear protein that shares a similar DNA-binding domain with the E. coli RecA protein [1]. Kin17 is ubiquitously expressed, with the highest expression levels in muscle, heart and testis [2]. The solution structure of the region 51-160 of Kin17 has been reported [3]. This fragment binds to double-stranded, curved DNA fragments located in illegitimate recombination sites [4-6]. DNA injury, such as ionizing radiation elevates Kin17 expression in mammalian cells [7]. Kin17 participates in the cellular response to genotoxic agents by acting as a DNA maintenance protein that helps to overcome the perturbation of DNA replication produced by unrepaired lesions [8, 9]. Kin17 is a component of the DNA replication complex relevant to cell cycle progression [10, 11], and it may be directly involved in the RNA splicing process [12, 13]. We have previously reported that up-regulation of Kin17 is strongly associated with cell proliferation, DNA replication, and DNA repair in breast cancer. Increased levels of Kin17 were shown in immortalized cells, and was associated with tumorigenesis [14].

Oral squamous cell carcinoma (OSCC) is an aggressive malignancy, which comprises more than 90% of all malignant epithelial tumors arising in the oral cavity. The 3-year survival rate for patients with advanced-stage OSCC treated with standard therapy is about 30%–50% [15]. Advances in combination treatments have contributed to the improvement of cancer therapy in the last three decades [16]. However, resistance to standard chemotherapy continues to be a limiting factor in the treatment of OSCC. There is a great need to clarify the mechanisms of chemoresistance in order to develop new chemotherapeutic strategies for OSCC. Since most

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chemotherapeutic drugs exert their toxicity to tumor cells primarily through induction of DNA damage, which blocks cell cycle and induces cell death, we hypothesized that Kin17 could be involved in chemoresistance of OSCC.

Previous studies have demonstrated various intracellular functions of Kin17. However, the extracellular activities of Kin17 have not yet been described. Therefore, we investigated Kin17 expression in OSCC tissue specimens, OSCC cell lines, and in human serum and cell culture supernatants to explore the functions of a secretory Kin17 protein. We demonstrate for the first time that the secretory Kin17 protein is produced by normal human epithelial cells, as well as by OSCC cells. Further, we show that the secretory Kin17 protein is necessary for the DNA damage response in OSCC.

MATERIALS AND METHODS

Tissue Specimens and Blood Samples

54 specimens were collected from the Department of Oral and Maxillofiacial Surgery, Hospital of Stomotology, Sun Yat-sen University and the Second Affiliated Hospital of Guangzhou Medical University. Samples were fixed in 10% formalin and prepared for embedding. Blood sera of health adults and OSCC patients were obtained from the Second Affiliated Hospital of Guangzhou Medical University. Collection of clinical data and samples was performed in accordance with the guidelines of the institutional review board.

Immunohistochemistry

Deparaffinized sections (4μm-thick) were immersed in 3% H_2O_2 for 30 min at room temperature to block endogenous peroxidase activity, following by incubation with 5% normal goat serum. After incubation with anti-Kin17 antibody (1:100, Santa Cruz) overnight at 4°C, the sections were then incubated with HRP-conjugated anti-mouse immunoglobulin (Dako) for 45 min, and DAB products were visualized according to the instructions of the Envision^{TM} Detection Kit (DAKO). Expression of Kin17 was viewed by Nikon microscope (Eclipse Ti-U). A semi-quantitative method was used to evaluate the degree of immunostaining ranging from “0” (no expression), “1” (very weak), “2” (weak to moderate), “3” (moderate to strong) to “4” (very strong) on a cell-by-cell basis in 5 microscopic fields (200x magnification).

Cell Culture

OSCC cell lines SCC25 and CAL27 were purchased from American Type Culture Collection (ATCC, Manassas, VA). KV cell line was obtained from Center of Experimental Animal of Sun Yat-sen University. All OSCC cell lines were maintained in DMEM/F12 (DF, Sigma) supplemented with 10% FBS (Hyclone). Normal mucosal epithelial cells were primary cultured in MCDB153/RPMI containing serum-free supplementaries described previously [17]. All cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Protein Preparation and Western Blot (WB)

The cells were lysed on ice for 1 hr with RIPA buffer (50mM HEPES pH7.5, 1% Triton X-100, 150mM NaCl, 100mM NaF) containing protease inhibitor cocktail (Roche). Conditioned media were concentrated to 100-fold at 5000×g using centrifugal filter devices (Millipore). Supernatant protein of OSCC cells was treated with PNGaseF (NEB) or Endo-N-Acetylgalactosaminidase (NEB) for 4 hr at 37°C, the deglycosylation results were detected by WB. For brefeldin A (BFA, Sigma) treatment, the cells were treated with different concentrations of BFA or 0.1% DMSO as a vehicle control for 12 hr, and conditioned media were then collected and concentrated. Protein concentrations were determined using the Protein Quantitative Analysis kit (K3000-BCA, Shenergy Biocolor).

Samples were separated in 10% SDS-PAGE gels and transferred onto PVDF membranes (GE Healthcare). Membranes were blocked with TBST containing 5% non-fat milk for 2 hr at room temperature and incubated with anti-Kin17 antibody (1:250, Santa Cruz) overnight at 4°C. Thereafter, membranes were incubated with HRP-conjugated anti-mouse IgG (1:5000, GE) for 1 hr at room temperature, and exposed with Supersignal^{R} West Pico Chemiluminescent Substrate (Pierce). Optical densities of western blot signals were measured using ImageQuant TL 7.0 Image Analysis Software (GE).

Expression and Purification of (His) 6-HAS-kin17

The ORF of (HAS) Kin17 cDNA was constructed into vector pET32a (Novagen), the resulting plasmid coding (His) 6-HAS-kin17 protein of 65.7KDa was fused with a 109aa thioredoxin protein. Bacteria were grown in LB medium plus 100μg/ml ampicillin up to

OD_{600}=0.6, then 1mM IPTG was added to the cultures and incubated for 4 hr at 20°C. The bacteria were harvested and resuspended in buffer (20mM Na_2HPO_4/NaH_2PO_4, 500mM NaCl, 20mM imidazole, pH7.4). The purification of recombinant protein was performed by using an affinity chromatography (on a chelating column with Ni^{2+}ions, Qiagen) and a cationic exchange chromatography (on a G25 column, GE). Thereafter, the purified recombinant protein was detected by WB and mass spectrometry (UltraflexIII, Bruker Daltonics).

CCK-8 Assay

The cells were seeded onto 96-well culture plate at 5000 cells/well, and were treated with 5-fluorouracil (5-FU) or cisplatin (CDDP) at different concentrations for 24 hr. (His) 6-HAS-kin17 was then added to the medium for 72 hr. Thereafter, CCK-8 was added and incubated for 4 hr at 37°C. OD_{450} was measured using microplate reader (BioTek). All experiments were independently repeated at least 3 times.

RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) followed by DNase I (Roche) digestion. Reverse transcription was performed using cDNA Synthesis Kit (TOYOBO), and the fragment of ABCC1 and ABCG2 was amplified using PCR system (Eppendorf), G3PDH was used as a control. Optical densities of PCR bands were measured using ImageQuant TL 7.0 Image Analysis Software. Primers were as followed: ABCC1, 5′-CTTCTGGAGGAATT GGTTGTATAGAAG-3′ (forward), 5′-GGTAGACCCAGA CAAGGATGTTAGA-3′ (reverse); ABCG2, 5′-AGTTCCATGGCACTGGCCATA-3′ (forward), 5′-TCAGGTAGGCAATTGTGAGG-3′ (reverse); G3PDH, 5′-ACCACAGTCCATGCCATCAC-3′ (forward), 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse).

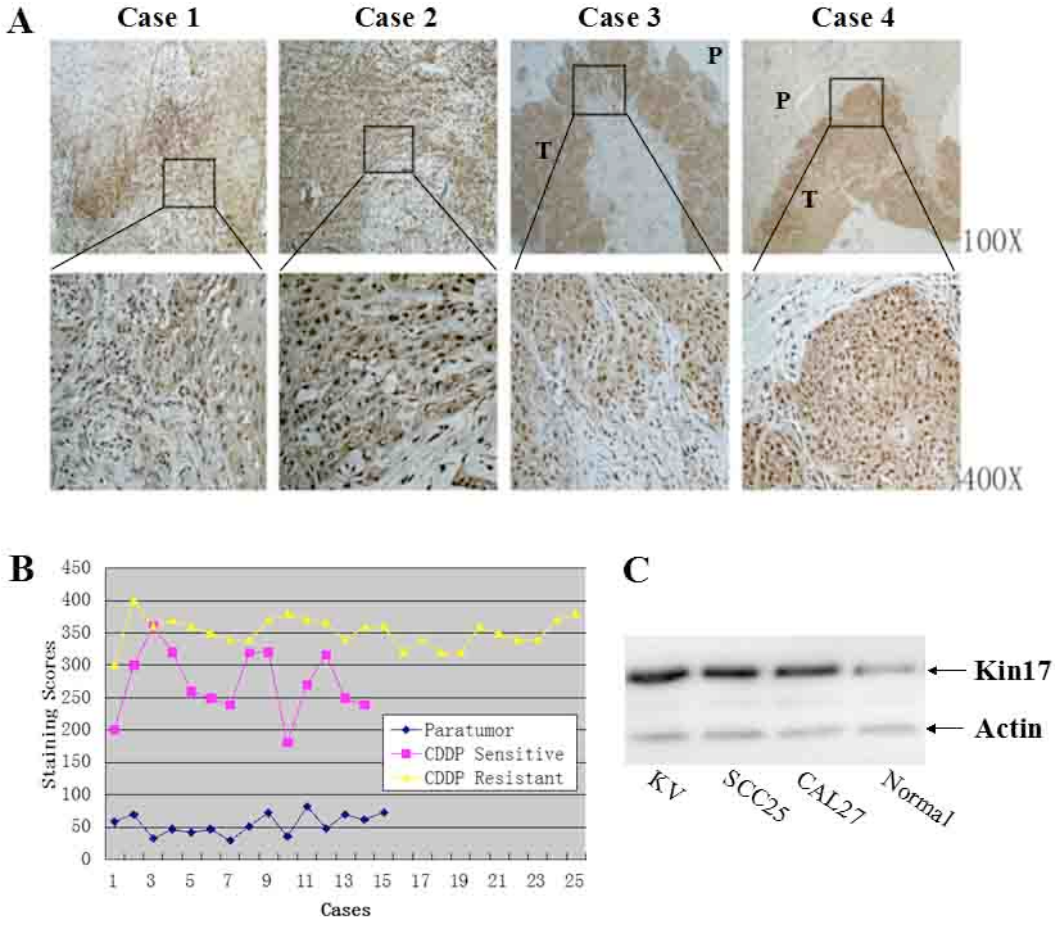

Figure 1: Kin17 expression in clinical OSCC specimens and cell lines.

(**A**) Immunohistochemical analysis of Kin17 expression in clinical OSCC tissues. Representative images are shown. T indicates tumor nest; P indicates paratumoral region. (**B**) Immunostaining scores of paratumoral tissues, the specimens of CDDP-sensitive group and the specimens of CDDP-resistant group. (**C**) WB analysis of Kin17 expression in normal mucosa epithelial cells and OSCC cells. 25μg of cell lysate was uploaded in each lane.

Statistical Analysis

Comparisons between groups were evaluated by a two-tailed student's t test and $p < 0.05$ was considered statistically significant.

RESULTS

Expression of Kin17 in OSCC Tissues and Cell Lines

We evaluated 54 specimens, including OSCC tissues and paratumoral tissues, for Kin17 protein expression using immunohistochemistry. OSCC tumor nests had significantly higher Kin17 expression compared to paratumoral tissues (Figure **1A**). Clinicopathological correlation did not reveal an association for Kin17 expression with tumor size, grade, metastasis, or with 5-year survival rates (data not shown). However, Kin17 expression was significantly higher in a patient group who received chemotherapy before surgery ($p<0.05$, Figure **1B**). Western blot (WB) analysis showed that Kin17 protein expression in OSCC cell lines was increased compared to normal epithelial cells (Figure **1C**).

Kin17 is an Unconventional Secretory Protein

We measured Kin17 protein expression in serum from patients with OSCC (n=24) and from a healthy population (n=10). Interestingly, Kin17 expression was observed in serum samples from a healthy population, as well as in serum from OSCC patients. However, it is noteworthy that serum protein expression of Kin17 was significantly higher in patients who received chemotherapy before surgery compared with healthy controls and patients who had not received chemotherapy (Figure **2A**). OSCC cell conditioned media was collected and concentrated to an optimal density (2mg/ml). Kin17 protein expression was measured by WB analysis (Figure **2B**). Our results indicated that Kin17 may be a secretory protein, although it has traditionally been characterized as a nucleic protein.

Using SignalP3.0 Server prediction, we did not find a signal peptide sequence within the Kin17 structural domain (Figure **2C**). Kin17 does contain two putative N-glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/) and three putative O-glycosylation sites

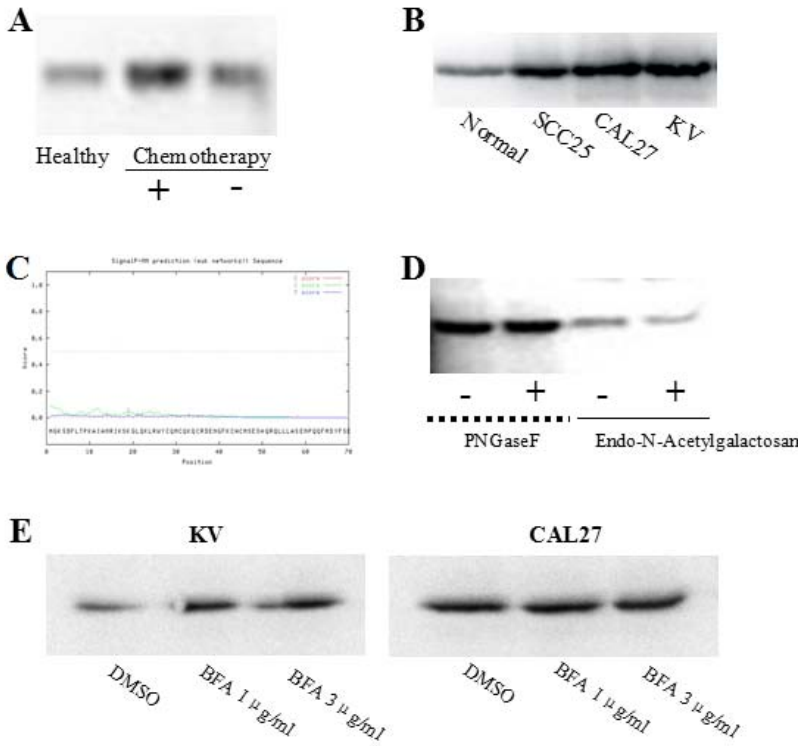

Figure 2: Kin17 expression in sera and cell culture supernatants.

(**A**) Kin17 expression was detected in blood sera of healthy adults or OSCC patients by WB. 1μl of each serum sample was 10-fold diluted and uploaded. Representative result is shown. (**B**) Visible signal was observed in the supernatants of normal mucosa epithelial cells and OSCC cells by WB. 30μg of protein was uploaded in each lane. (**C**) Prediction of signal peptides in Kin17 sequence was performed by Bioinformatics online. (**D**) Deglycosylation analysis of KV cell supernatants by WB. 30μg of protein was uploaded in each lane. (**E**) The cells were treated with BFA (1μg/ml or 3μg/ml), or 0.1% DMSO as a vehicle control for 12 hr, and conditioned media were then collected and concentrated. 30μg of protein was uploaded in each lane.

(http://www.cbs.dtu.dk/services/NetOGlyc/). However, when the conditioned media was pretreated with PNGaseF or Endo-N-Acetylgalactosaminidase, the supernatant proteins could not be deglycosylated, suggesting that Kin17 is not a glycoprotein (Figure **2D**).

Although Kin17 is lack of a classical signal peptide, it is possible that the protein may be exported through the conventional secretory pathway. Thus, there are four kinds of possibilities about Kin17 secretion: The first, secretory Kin17 is mediated by undefined post-translational modifications; the second, by other ways dependent upon a functional conventional secretion pathway; the third, by association with conventional secreted proteins; and the last, secretory Kin17 might be unconventional. To address this possibility, the cells were treated with well-characterized conventional secretion inhibitor, BFA, a blocker of ER-to-Golgi transport, or 0.1% DMSO for 12 hr. At harvest, cells were approximately 90% confluent and showed no visual evidence of cytotoxicity (data not shown). The results from WB analysis show that secreted Kin17 in two cell lines were not reduced by BFA (Figure **2E**), suggesting that Kin17 is secreted via unconventional secretory pathways.

Kin17 Improved Chemoresistance of OSCC

Consistent with its role in genomic stability, cellular expression of Kin17 increases when DNA damage is induced by UV or ionizing radiation. In order to investigate the association of the secretory Kin17 protein with DNA damage or DNA repair, we treated OSCC cells with 5-FU or CDDP at various concentrations for 48 hr. We found that the expression of secretory Kin17 protein, but not cellular Kin17 protein, increased significantly, indicating that the secretory protein is important for the DNA damage response in OSCC cells (Figure **3A**). We also observed that a novel 35KDa isoform of Kin17 emerged when OSCC cells were treated by chemotherapeutics drugs. The emergence of this isoform suggests that the 35KDa signal of OSCC cell supernatants might be especial cytotoxicity stress proteins, which induced by chemotherapy.

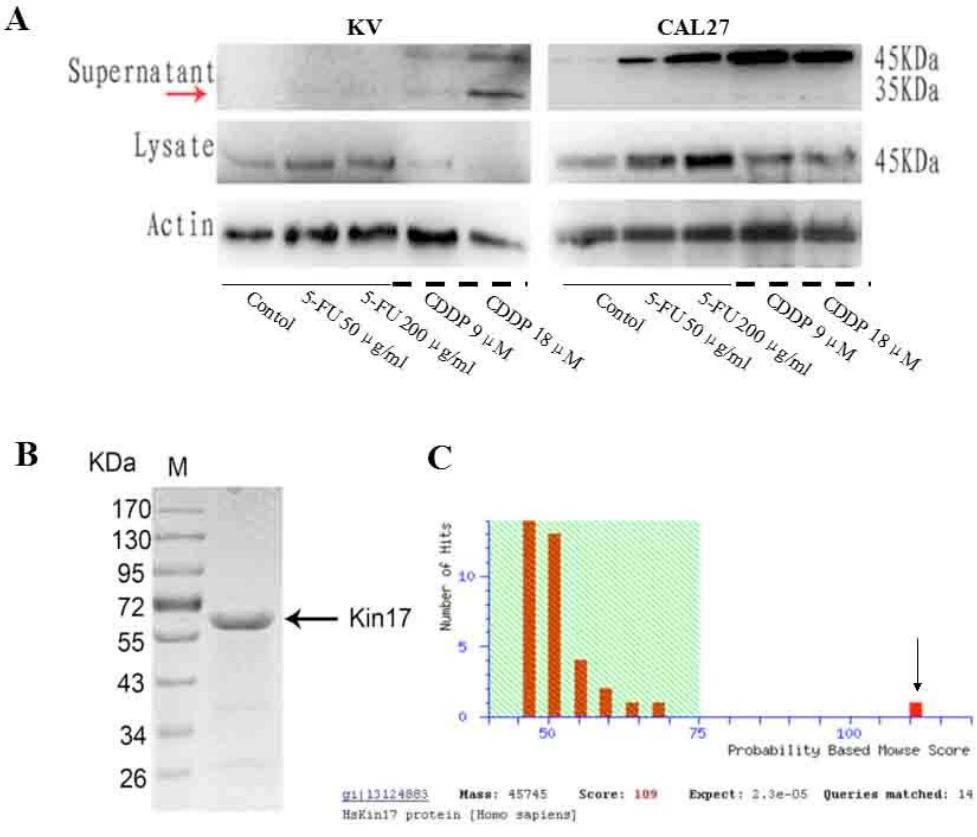

Figure 3: The effect of chemotherapy on Kin17 expression.

(**A**) OSCC cells were treated with 5-FU or CDDP at different concentrations, and then the supernatants or cellular proteins were collected and detected by WB. Arrow indicates 35KDa protein. 30μg of protein was uploaded in each lane, and β-Actin was used as a loading control. (**B**) The purified recombinant protein (His) 6-HAS-Kin17 was verified by 10% SDS-PAGE and coomassie brilliant blue staining. (**C**) Mass spectrometry analysis showed up human Kin17 protein. Arrow indicates the highest score hit.

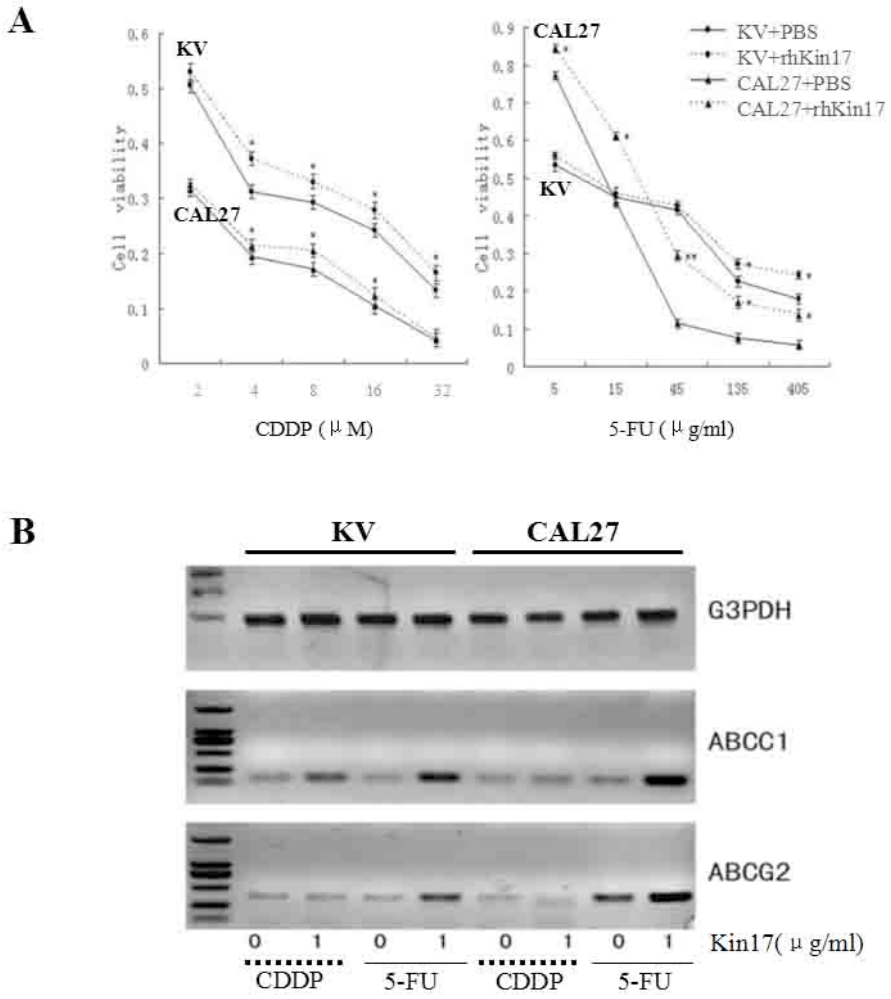

Figure 4: Effect of Kin17 on OSCC chemosensitivity.

(**A**) OSCC cells were treated with 1μg/ml rhKin17 for 72 hr followed by the administration of 5-FU or CDDP for 24 hr. Survival rate of OSCC cells was detected by CCK-8 assay. Error bars represent the SD, *, $p<0.05$, **, $p<0.01$. (**B**) OSCC cells were treated with 5-FU or CDDP for 12 hr, and then 1μg/ml rhKin17 was added to the medium for 24 hr. ABCC1 and ABCG2 mRNA expression level was detected using semiquantitative RT-PCR (28 cycles), G3PDH was used as a control.

We then constructed and purified a recombinant protein, (His) 6-HAS-Kin17, to analyze the function of the secretory Kin17 protein. (His) 6-HAS-Kin17 was verified by 10% SDS-PAGE (Figure **3B**) as a single band. We corroborated that the single band was human Kin17 protein by mass spectrometry (Figure **3C**). The recombinant human Kin17 protein (rhKin17) enhanced chemoresistance to 5-FU or CDDP in OSCC cells (Figure **4A**). Furthermore, rhKin17 significantly increased the transcriptional expression of the ATP-binding cassette (ABC) transporters ABCC1 and ABCG2, which have been implicated in multidrug resistance (Figure **4B**).

DISCUSSION

In this study, we found that Kin17 was expressed in human serum, normal oral epithelial cell supernatant and OSCC cell supernatants as a secretory protein. We also found a significant association between the secretory Kin17 protein and OSCC chemoresistance.

Chemotherapy resistance is one of the most challenging problems in OSCC treatment [18]. It has been reported that nearly 30% of newly diagnosed cases of OSCC and more than 70% of relapsed cases develop chemoresistance [19]. The molecular mechanisms mediating chemoresistance are widely studied, but still poorly understood [20, 21]. In the present study, we found strong Kin17 expression in clinical OSCC specimens that had exhibited chemoresistance, suggesting it plays a role in mediating resistance to chemotherapy drugs. We demonstrated that serum Kin17 levels were elevated in patients with OSCC who had received chemotherapy. We also showed that secretory Kin17, but not cellular

Kin17, increased *in vitro* after OSCC cells were treated with 5-FU or CDDP. Thus, our study demonstrates for the first time that Kin17 functions as a secretory protein in OSCC, and that chemotherapy increases its expression.

The Kin17 protein is ubiquitously expressed in nearly all human tissues and organs [2, 22]. Although Kin17 displays a zinc finger motif and a nuclear localization signal similar to those observed in other nuclear proteins [23], Kin17 lacks some conventional secretory protein characteristics, such as an amino-terminal signal peptide sequence and post-translational glycosylation [24-27]. We found that the secretory Kin17 protein could be activated by chemotherapy, indicating its role as a cytotoxicity stress protein in the DNA damage response. We conjectured that translocation of Kin17 may occur via an exosome, a monoptychial vesical fused with a multivesicular body and cell membrane [28]. The representative protein of exosome secretion is the heat-shock protein 70, which participates in the immune response [29].

To better understand the role of the secretory Kin17 protein, we constructed and purified rhKin17. When OSCC cells were treated with rhKin17, the cells became more resistant to 5-FU or CDDP. In addition, rhKin17 treatment promoted the expression of multidrug resistance transporter proteins in OSCC cells. Multidrug transporter proteins, such as the ABC transporters ABCB1, ABCC1, ABCC2 and ABCG2, are well known for their contribution to chemoresistance through the efflux of cytotoxic drugs from cancer cells [30]. ABCC1 (Multidrug resistance-related protein 1, MRP1), a membrane-bound, energy-dependent efflux transporter, is overexpressed in several kinds of multidrug resistant cell lines and related to multidrug resistance (MDR) of various cancers. Cai *et al*. showed that down-regulation of ABCC1 in drug-resistant mucoepidermoid carcinoma increased chemosensitivity to 5-FU and CDDP [31]. Moreover, ABCG2 is believed to be a reliable marker for subpopulations of cells with chemoresistance [32, 33]. To our knowledge, this study is the first to report that Kin17 regulates ABC transporter transcriptional expression and chemosensitivity. We show that rhKin17 administration decreased chemotherapy-induced cytotoxicity and enhanced the ability of OSCC cells to facilitate DNA damage repair. Therefore, the effective inhibition of Kin17 secretion could preferably inhibit tumor progress and help maintain chemotherapeutic effectiveness. Secretory proteins play an important role in the immune response and in hormone and enzyme activation. A role for a secretory protein in the DNA damage response is an important finding in the present study, although the underlying mechanisms remain unclear. Further research on the effects of the secretory Kin17 protein on physiological or pathological processes is warranted.

In conclusion, our study provides evidence that Kin17 is a secretory protein with some unconventional characteristics. The secretory Kin17 protein may play a role in chemoresistance in OSCC. Together, this study and previous findings support the notion that targeting Kin17 is an important future direction for OSCC therapy.

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