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## Anti-EGFR antibody cetuximab is secreted by oral squamous cell carcinoma and alters EGF-driven mesenchymal transition

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### ABSTRACT

Genetic amplification, overexpression, and increased signaling from the epidermal growth factor receptor (EGFR) are often found in oral squamous cell carcinoma (OSCC) and thus EGFR is frequently targeted molecularly by the therapeutic antibody cetuximab. We assessed effects of cetuximab in control of EGF-driven malignant traits of OSCC cells. EGF stimulation promoted progression level of mesenchymal traits in OSCC cells, which were attenuated by cetuximab but incompletely. We pursued a potential mechanism underlying such incomplete attenuation of OSCC malignant traits. Cetuximab promoted secretion of EGFR-EVs by OSCC cells and failed to inhibit EGF-driven secretion of EGFR-EVs. Cetuximab was also found to be robustly secreted with the EGFR-EVs by the OSCC cells. Thus, EGF promotes the level of mesenchymal traits of OSCC cells and secretion of EGFR-EVs, which involve cetuximab resistance.

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### 1. Introduction

The EGF receptor (EGFR/ErbB1/Her1) is a member of ErbB/EGFR receptor family and is one of the crucial transforming receptor tyrosine kinases (RTK) in head and neck squamous cell carcinoma (HNSCC), including oral squamous cell carcinoma (OSCC) [1]. Incidence and progression of HNSCC is correlated with habitual usage of tobacco, leading to release of nicotine [2]. Nicotine then triggers secretion of EGF, which binds to EGFR and activates the PI3K-AKT, RAS-MEK-ERK, and JAK-STAT signaling pathways, thus promoting proliferation and survival of cancer cells [3]. In addition, activating genetic aberrations such as amplification and mutation of EGFR are

found in approximately 15% of human papillomavirus-negative [HPV (–)] HNSCC such as OSCC [1].

The molecular targeting drug cetuximab (cmab, Erbitux<sup>®</sup>) is an anti-EGFR IgG<sub>1</sub> monoclonal antibody effective in the therapy of HNC locoregionally advanced or recurrent/metastatic HNSCC and KRAS wild-type, EGFR-expressing metastatic colorectal cancer [4,5]. Cetuximab has 5-fold higher affinity for EGFR compared to EGF, and thus is able to block EGFR-ligand interaction, inhibit dimerization of EGFR, and inhibit downstream RAS-MEK-ERK signaling pathway [6]. However, several resistance mechanisms to this therapeutic antibody have been found [7].

We recently showed that OSCC cells secrete EGFR-containing extracellular vesicles (EGFR-EVs) and this EGFR-EV-secretory phenotype was more prominent in lymph-node-metastatic OSCC cells [8]. Extracellular vesicles (EVs) are secreted structures surrounded by lipid bilayer membranes containing a variety of molecular cargoes [9–13]. According to the secretory mechanism and vesicle size, EVs are classified as exosomes (30–200 nm),

*Abbreviations:* Cmab, cetuximab; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; EV, extracellular vesicles; HNSCC, head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma.

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microvesicles (MVs) (100–1000 nm), apoptotic bodies (1000 to 5000 nm), and matrix vesicles (found in extracellular matrices and carrying abundant minerals) [14–17]. EVs, in particular exosomes usually contain tetraspanins including CD9 [18]. We recently showed that OSCC cells secrete EGFR-EVs as well as heat shock protein-enriched EVs (HSP-EVs) and this EV-secretory phenotype was more prominent in lymph-node-metastatic OSCC cells [8]. In addition, EpCAM-EVs were abundantly secreted by highly resistant prostate cancer cells [19]. Recently, it has become clear that EVs play a key role in cell-to-cell communication and participate a range of biological events [20,21]. Particularly, the “seed and soil” theory has been advocated in the field of cancer EVs [22]. EVs secreted by cancer cells can educate the local tumor milieu, while small vesicles such as exosomes can reach to and influence distant organ milieu via the circulation, indicating a role in influencing the “soil” component [23] [24]. These significant properties of tumor-derived EVs prompted us to ask whether EGFR-EVs secreted by OSCC cells could affect surrounding normal squamous epithelial cells within the tumor milieu.

The epithelial-to-mesenchymal transition (EMT) is a cellular transformation, important in many aspects of cell biology, which involves a switch from an epithelial to mesenchymal shape [25,26]. Normal epithelial cells are tightly connected to each other through intercellular adhesion, cell junctions and desmosomes and loss of these connections is accompanied by altered cellular shapes, increased motility, and EMT [27]. Pre-cancerous cells often exhibit EMT, promoting migration and invasion of the cells within the tumor milieu [28]. EMT is a complex process consisting of multiple sequential steps and pathways [28]. It has been shown that cancer cells can be reprogrammed to new phenotypes by features in the tumor milieu, changes including transition between EMT and its reverse process MET [19,29]. Recent studies have shown that extracellular vesicles, such as exosomes and microvesicles, can trigger cell differentiation and transformation through transferring oncogenic components to recipient cells [30,31].

These studies prompted us to investigate potential roles for anti-EGFR antibody cetuximab in control of EGF-driven progression of mesenchymal traits in OSCC cells that secrete EGFR-EVs.

## 2. Methods

### 2.1. Cells

The human oral squamous cell carcinoma cell line HSC-3 was obtained from JCRB Cell Bank at National Institutes of Biomedical Innovation, Health, and Nutrition. The human oral squamous (epithelial) cell line RT7 was provided by Dr. Nobuyuki Kamata (Hiroshima University, Japan). HSC-3 cells were cultured in DMEM supplemented with 10% FBS for maintenance and in serum-free DMEM for preparation of EVs. RT7 cells were cultured in KGM-Gold or KGM-2 supplemented with recombinant human EGF (0.125 ng/ml), bovine pituitary extract (0.004 ml/ml), recombinant human insulin (5 µg/ml), hydrocortisone (0.33 µg/ml), epinephrine (0.39 µg/ml), human holo-transferrin (10 µg/ml), CaCl<sub>2</sub> (0.06 mM), gentamycin and amphotericin B (Lonza, Basel, Switzerland).

### 2.2. Reagents

Recombinant human EGF (carrier free) (585506, BioLegend, San Diego, CA). Cetuximab (Erbix<sup>®</sup> Injection, MerckSerono, Tokyo, Japan).

### 2.3. Preparation and analysis of EVs

EVs were prepared using a polymer-based method with

modifications [8]. Cells cultured were washed with Hanks' balanced salt solution (HBSS), and then further cultured in serum-free medium for 2 days. Cell culture supernatant was centrifuged at 2000 × g for 30 min at 4 °C to remove detached cells. The supernatant was then centrifuged at 10,000 × g for 30 min at 4 °C to remove cell debris. The concentrate was applied to a polymer method using Total Exosome Isolation (ThermoFisher Scientific). The EV fractions were eluted in 100 µl PBS (–). For protein assay, 10 × RIPA buffer containing 10% NP-40, 1% SDS, and 5% deoxycholate in PBS (–) and an EDTA-free protease inhibitor cocktail (Sigma) were added to the EV fraction, incubated on ice for 15 min and applied to micro BCA protein assay system (ThermoFisher Scientific). Transmission Electron Microscopy (TEM) was carried out as described [8,19] with 20,000 times magnification using an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at Central Research Laboratory, Okayama University Medical School. Particle diameter distribution was analyzed using a Zetasizer as described [8].

### 2.4. Western blotting analysis

Protein samples from the EV and cellular fractions were prepared as described [8,19]. For analysis of CD9, protein samples were mixed with an SDS sample buffer without any reducing agent and boiled. For analysis of cetuximab, both reduced and non-reduced conditions were tested. Otherwise, protein samples were mixed with the SDS sample buffer containing β-mercaptoethanol and then boiled. Protein samples were separated by SDS-PAGE in 4–20% TGX-GEL (BioRad) and transferred to PVDF membranes by using semi-dry or tank method. The antibodies used were mouse anti-CD9 (1:1000, MBL), anti-EGFR (1:1000, abcam), rabbit anti-vimentin (1:1000, D21H3, CST), rabbit anti-E-cadherin (1:1,000, 24E10, CST), rabbit anti-HSP 90β (1:1000, GeneTex, Irvine, CA), HRP-conjugated goat anti-human IgG (H + L chain) (1:5,000, MBL) for detection of cetuximab, and rabbit anti-GAPDH (1:5000, D16H11, CST).

### 2.5. Cell morphology analysis

Phase contrast cell images of random three fields were taken using Olympus CK30 equipped with a Digital Microscope Eyepiece Model MA88 (Premiere<sup>®</sup>). Spindle shaped cells were defined as the cells with more than two-fold length/width ratio as described [32].

### 2.6. Cancer gene panel analysis

Genomic DNA was isolated from HSC-3 cells using DNeasy Blood & Tissue kit (Qiagen) and analyzed with TruSight Tumor 15 (Illumina, San Diego, CA).

### 2.7. Statistical analysis

Statistical significance was calculated using GraphPad Prism software. Three or more mean values were compared using one-way analysis of variance (ANOVA) with pair-wise comparisons by the Tukey's multiple comparison test. Data were expressed as the mean ± S.D.

## 3. Results

### 3.1. Cetuximab altered EGF-driven progression of EMT in OSCC cells

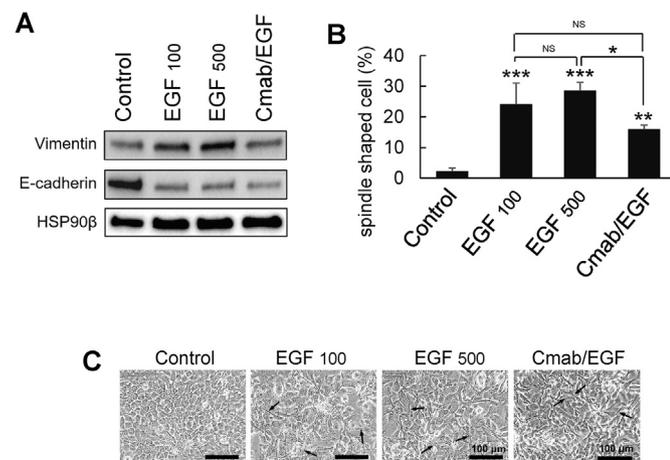
Cells undergoing EMT are characterized by change in their shapes to a spindle-like morphology, reduced levels of intercellular adhesion molecules such as cadherins and increases in

mesenchymal markers such as vimentin [19,26,33]. We assessed whether EGF could alter progression of EMT in OSCC cells, which could be reversed by cetuximab. We examined expression of vimentin, a marker of the mesenchymal transition and E-cadherin, a marker of epithelial adhesion. EGF stimulation increased levels of vimentin and reduced the levels of E-cadherin in the OSCC cells (Fig. 1A). Exposure to cetuximab attenuated the EGF-dependent vimentin expression although it did not restore the levels of E-cadherin in the OSCC cells (Fig. 1A). These findings indicated that the EGF caused the OSCC cells to undergo progression towards EMT, a change that cetuximab partially attenuated. Coincidentally, the EGF stimulation increased the number of cells with spindle shapes in the OSCC cells (Fig. 1B and C). Cetuximab partially inhibited such EGF-driven morphological changes, although this effect was incomplete (Fig. 1B and C). These finding indicated that cetuximab attenuated EGF-driven progression of EMT in OSCC cells while potential cetuximab resistance of OSCC cells was implicated.

### 3.2. EGF and cetuximab promoted secretion of EGFR-EVs by OSCC cells

A definition of EVs is that they are particles surrounded by lipid bilayers, although the sizes of EVs, including exosomes and MVs, depend on the cell of origin and other contexts [34,35]. We showed that EVs secreted by OSCC cells contained exosome marker CD9 and EGFR while the size of the EVs are between 50 and 200 nm, which could contain exosomes and microvesicles [8]. Hence, we use the term “EVs” in the present study as well. We examined morphology and size of the EVs secreted by the OSCC cells treated with or without EGF. The EVs analyzed under transmission electron microscopy (TEM) appeared to be surrounded by lipid bilayers and were sized at approximately 50–200 nm in either EGF-stimulated or unstimulated conditions (Fig. 2A). Their particle diameter distribution peaked between 150 and 230 nm in either EGF-treated or untreated conditions (Fig. 2B).

We next assessed whether secretion levels of EGFR-EVs by the OSCC cells were altered in response to EGF and/or cetuximab.



**Fig. 1.** Stimulation of EGF promoted progression level of mesenchymal traits in OSCC cells, attenuated by cetuximab incompletely. HSC-3 cells were administrated with or without 10  $\mu$ g/ml of cetuximab (Cmam) 30 min before EGF stimulation (0, 100 or 500 nM) in the serum-free condition for 48 h. (A) Western blot showing Vimentin and E-cadherin with or without EGF and Cmam. Cells were administrated with or without 10  $\mu$ g/ml of Cmam and 100 nM of EGF. HSP90 $\beta$ , loading control. (B) Rate of spindle-shaped cells. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P = 0.016$ ,  $n = 3$ . NS, not significant. (C) Representative phase-contrast photomicrographs of HSC-3 cells cultured with or without Cmam or EGF. Scale bars, 100  $\mu$ m. Arrows indicate projections of spindle-like cells.

Notably, secretion of EGFR-EVs by oral cancer cells was promoted by the administration of either EGF, cetuximab or their combination (Fig. 2C). Cetuximab did not inhibit the EGF-driven secretion of EGFR-EVs but co-stimulated it (Fig. 2C). These finding suggested that cetuximab first bound with EGFR and cetuximab/EGFR-EVs were secreted. Simultaneously with such EGF-driven secretion of EGFR-EVs, cellular EGFR levels were profoundly reduced (Fig. 2C). Conversely, cetuximab, which competes with EGF, appeared to bind to and retain cellular EGFR within cells, but also promoted secretion of EGFR-EVs (Fig. 2C). These findings indicated that EGF and cetuximab stimulated secretion of EGFR-EVs by OSCC cells.

### 3.3. The anti-EGFR antibody cetuximab is secreted by OSCC cells

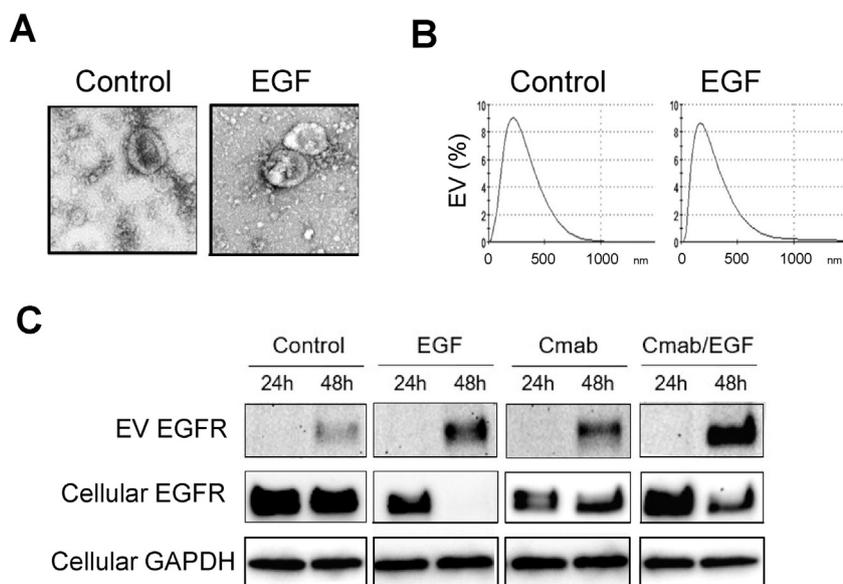
According to these findings, we made a working hypothesis that the OSCC cells were able to secrete cetuximab within EGFR-EVs, a property which could void a potential neutralization role of cetuximab to the EGF-EGFR signaling. We therefore examined whether cetuximab was secreted with the OSCC-EVs by the OSCC cells. Cetuximab was detected in the EV fraction that contained CD9 (Fig. 3). The concentration of cetuximab in the EV and cellular fractions increased with the dose-dependent manner of the cetuximab added to the OSCC cells (Fig. 3). Coincidentally, intracellular CD9 level was increased with cetuximab concentration dependent manner. These findings indicated that OSCC cells could secrete EGFR-EVs and cetuximab. Cetuximab-bound EGFR could be retained in OSCC cells yet simultaneously OSCC cells can secrete cetuximab/EGFR-EVs.

### 3.4. Genetic alterations in the OSCC cells

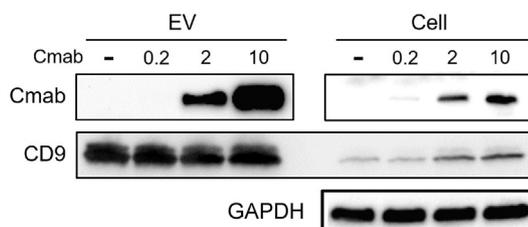
To exclude the possibility that any activating mutations in the RAS-MEK-ERK signaling pathway participates in EGF-dependent and/or cetuximab-dependent EGFR-EV secretion by oral cancer cells, we examined genetic aberrations in the HSC-3 cells by using cancer gene panel analysis. A nonsense mutation TP53 R306\* was detected with frequency value 0.935 by performing upon full coding DNA sequencing (CDS) of TP53. However, neither potential mutations in EGFR, ERBB2, KRAS, NRAS, BRAF, PIK3CA, PDGFRA, KIT nor focal amplification in MET were found in the HSC-3 OSCC cells. We next confirmed mutations occurred in HSC-3 oral cancer cells by searching the cancer cell line encyclopedia (CCLE) [36]. Frame-shift insertions in PRKDC (encoding DNA-dependent protein kinase), a nonsense mutation in CASP8 (encoding caspase 8), missense mutations in SMAD4, CLTCL1 (encoding clathrin heavy chain) and PCDH15 (encoding proto-cadherin) were found in the HSC-3 cells (Table S1). Thus, although alterations were seen in some pathways, these findings implied a minimal role for activating mutation in RTK/RAS/RAF/PI3K signaling pathways and suggested other genetic aberrations involve the observed transforming vesicles generated by the malignant phenotype of the HSC-3 OSCC cells.

## 4. Discussion

Stimulation of EGF promoted progression level of mesenchymal traits in OSCC cells, which were attenuated by cetuximab but not completely (Fig. 1). We pursued a mechanism underlying such incomplete attenuation. Cetuximab promoted secretion of EGFR-EVs by OSCC cells and failed to inhibit EGF-driven secretion of EGFR-EVs (Fig. 2). It was thus suggested that cetuximab could alter trafficking of EGFR-EVs toward their secretion. Finally, cetuximab was found to be secreted with the EGFR-EVs by the OSCC cells (Fig. 3). Hence, it was suggested that secretion of cetuximab with EGFR-EVs by OSCC cells is a mechanism underlying cetuximab resistance of OSCC cells. EGF was shown previously to trigger EGFR



**Fig. 2. Cetuximab and EGF promoted secretion of EGFR-EVs by OSCC cells.** (A) TEM analysis of OSCC-EVs. EVs were prepared from HSC-3 cells treated with or without 100 nM EGF. Scale bars, 200 nm. (B) EV diameter distribution analysis. Similar results were obtained from two independent experiments. (C) Western blot showing EGFR in EVs secreted by HSC-3 OSCC cells. EVs and whole cell lysates were prepared from HSC-3 cells treated with or without 100 nM EGF and 10  $\mu$ g/ml of cetuximab in serum-free media for 24 or 48 h. Similar data were obtained from two independent experiments.



**Fig. 3. Secretion of cetuximab with EVs by OSCC cells.** HSC-3 cells were treated with cetuximab at concentrations of 0, 0.2, 2 or 10  $\mu$ g/ml for 2 days in KGM-2 medium. Western blot is showing cetuximab and CD9 in EVs and HSC-3 cells. GAPDH, loading control. Similar data were obtained from two independent experiments.

dimerization and internalization into endosomes. The generation of EGFR-EVs appears to be an alternative mechanism, involving entry of lipids into multivesicular bodies (MVBs), fusion of such bodies with plasma membranes and release of EVs. Furthermore, EGFR-EVs can be directly processed out from plasma membranes. Indeed, EGF stimulation triggered loss of cellular EGFR and gain of EGFR-EVs (Fig. 2C). Thus, the data suggested that EGF triggers endosome-mediated degradation of EGFR as well as secretion of EGFR-EVs.

Genetic aberrations in somatic cells underlie tumor incidence and cancer progression. Amplification and activating mutation in oncogenes, as well as inactivating mutation and deletion of tumor suppressor genes, can initiate and promote cancer progression [37]. Genetic aberrations in the EGFR gene, including amplification and mutation, are found in approximately 15% of human papillomavirus-negative [HPV (-)] head and neck squamous cell carcinoma [1]. Overexpression of EGFR was seen in the HSC-3 oral squamous carcinoma cells as compared to RT7 oral squamous cells (unpublished data), suggesting that the EGFR gene can be amplified and thus overexpressed in the HSC-3 cells. Indeed, EGFR-EVs were abundantly secreted by the HSC-3 OSCC cells and thus effectively bound to cetuximab. Several mechanisms of drug resistance to EGFR-targeted therapy have been demonstrated [38]. We classify these resistance mechanisms as follows: The first resistance class is

the amplification and activation of alternative RTK, e.g. ErbB/EGFR family, IGF1R, PDGFR $\beta$ , FGFR, MET, and the EGFR-S492R mutation that inhibits cetuximab binding [38]. The EGFR gene can be amplified in the HSC-3 OSCC cells, which overexpressed EGFR, potentially contributing cetuximab resistance. The second resistance class is through activating mutations in intracellular signaling proteins such as PIK3CA, RAS family (KRAS-Q61K or NRAS), BRAF-V600E, MEK (C121S or P124L) [38]. These mutations have been found in colorectal cancer, lung cancer and melanoma. Among these, activating mutations in PIK3CA have been often detected in HNC [1]; however none of these mutations were not found in the HSC-3 OSCC cells (Table S1). The third resistance class involves stromal signals, including HGF-high stroma that activates HGF-MET signaling [38,39]. We have previously shown that HGF-MET signaling can be driven under HSF1-HSP regulation in breast cancer [39]. HGF-high stroma in OSCC cases is under intense investigation. In addition to these recognized mechanisms of drug resistance, we have shown that highly-resistant cancer stem-like cells robustly secrete EpCAM-EVs [19]. Here we suggest a fourth class of the drug resistance involving vesicle-mediated secretion of the molecularly targeted anti-EGFR antibody which leads to protection of cancer cells (Fig. 4).

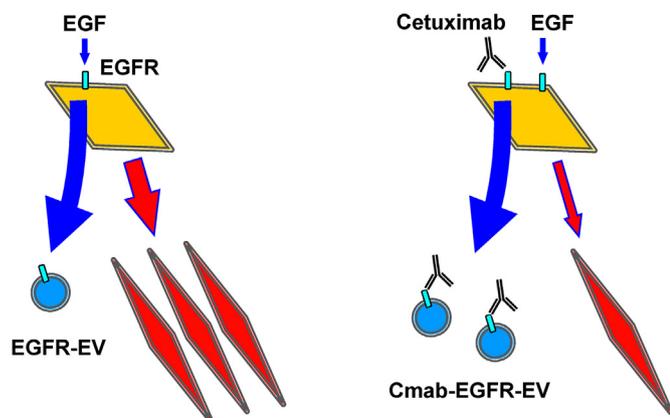
In conclusion therefore, we have demonstrated that OSCC-derived EVs trigger the secretion of a molecularly targeted antibody drug cetuximab, whose secretion could be a novel mechanism underlying antibody drug resistance in OSCC.

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#### Author contributions

TE and KK conceptualized and designed the study. KK, TE, CS, TF, JA prepared resources and devised methodology. TF, CS, KiO carried out the experimentation. TE, TF interpreted data. TE wrote the



**Fig. 4.** Summary. EGF stimulation of OSCC cells increased secretion of EGFR-EVs and level of EMT (left). Cetuximab attenuated EGF-driven progression of EMT but incompletely (right). Coincidentally, cetuximab was secreted with EGFR-EVs by OSCC cells, implicating a mechanism underlying incomplete inhibition of EMT and cetuximab resistance.

manuscript. SKC, SI, JM, KuO, TE revised and edited the manuscript. All authors reviewed the manuscript.

### Conflicts of interests

The authors have no conflict of interests to declare.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bbrc.2018.07.035>.

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