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PTEN Is Activated by the Addition of Cetuximab to Paclitaxel in Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim: The mechanisms through which cetuximab (cMab) coadministration with paclitaxel (PTX) enhances antitumor efficacy remain unclear. We examined the mechanism of the antitumor enhancing effect of cMab by determining changes in gene expression in the PI3K-AKT pathway. Materials and Methods: Eight human oral squamous cell carcinoma (OSCC) cell lines were cultured three-dimensionally and exposed to PTX + cMab. The expression levels of PTEN mRNA in OSCC cell lines after anticancer drug treatment were assessed using real-time PCR. PTEN mRNA expression levels were also confirmed after administration of PTX + cMab in vivo. Western blot analysis was used to confirm the results at the protein level. Results: PTEN mRNA and protein expression were significantly increased only in the cell lines with high sensitivity to PTX + cMab, and similar results were observed in vivo. Conclusion: PTEN activation may enhance the antitumor effect of PTX + cMab.*

Recent studies have reported the remarkable benefits of coadministration of paclitaxel (PTX), a taxane anticancer drug, and cetuximab (cMab), an anti-human epidermal growth factor receptor (EGFR) antibody, for the treatment of recurrent/metastatic oral cancer (1). In particular, the response rate to PTX+cMab therapy has been reported to be 60% in patients whose disease had progressed after second-line treatment for platinum-refractory head and neck squamous cell carcinoma with nivolumab, an immune checkpoint inhibitor for human programmed cell death-1 (PD-1) (2). However, there are currently no clear biomarkers for predicting the effect of cMab and PTX coadministration and the mechanisms associated with the enhancement of antitumor effects.

The EGFR signaling pathway, which is the pathway targeted by cMab includes the RAS signaling pathway involved in the proliferation of cancer cells and the Phosphoinositide 3-kinase (PI3K)-AKT pathway involved in the anti-apoptosis, invasion, and migration of cancer cells (3, 4). The activation of the PI3K-AKT pathway starts with the phosphorylation of PI3K; subsequently, the phosphorylation of AKT promotes anti-apoptosis, invasion, and migration of cancer cells (4). Conversely, phosphatase and tensin homolog (PTEN) has been shown to inhibit cancer cell proliferation by inhibiting the PI3K-AKT pathway (5). Although many studies have shown that low PTEN expression is involved in cMab resistance (6-9), there are also reports that PTX resistance, and not just cMab resistance, is related to PTEN (10); therefore, there is a need to evaluate alterations of gene expression in the PI3K-AKT pathway including PTEN as a mechanism for the enhancement of antitumor effects with PTX+cMab. The collage gel droplet embedded culture drug sensitivity test (CD-DST) is an anticancer drug sensitivity test that combines a low-volume 3-dimensional (3D) culture, serum-free media, and an image colorimetric assay, and is effectively used to guide personalized treatment for lung, breast, gastric, and colorectal cancers (11); reportedly, it is also capable of evaluating the sensitivity of oral cancers to PTX+cMab (12). Furthermore, because the exposure concentration of each anticancer drug, including cMab, under physiological conditions has been established, confirming genetic changes because of exposure to anticancer drugs in conditions that are closer to *in vivo* conditions is feasible by performing 3D cultures in the collagen gel. In this study, we used eight OSCC cell lines to examine the presence or

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Key Words: Anticancer drug sensitivity test, CD-DST, paclitaxel, cetuximab, PTEN.

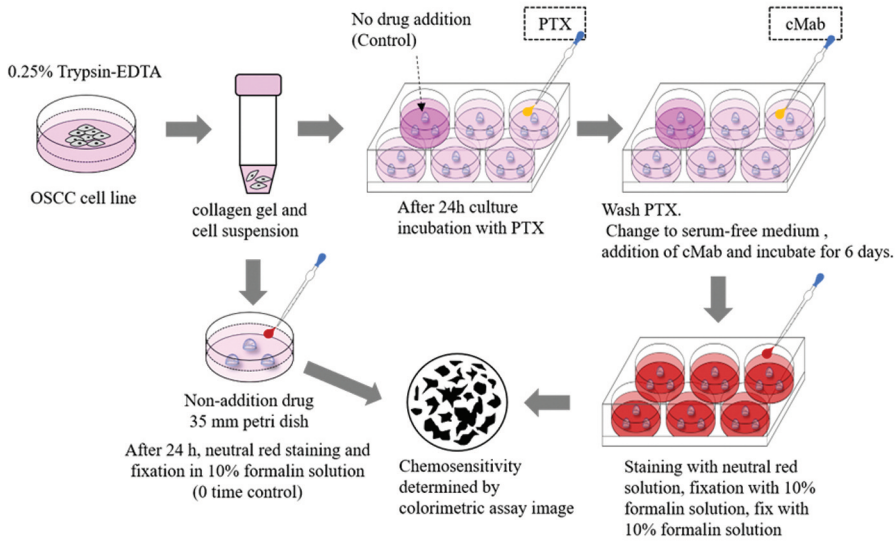

Figure 1. *Overview of the collage gel droplet embedded culture drug sensitivity test. Sensitivity of the eight oral squamous cell carcinoma (OSCC) cell lines to paclitaxel (PTX), cetuximab (cMab) and PTX+cMab was examined. High sensitivity was defined as T/C values of ≤50% and low sensitivity was defined as those of >50%.*

absence of KRAS mutations and their sensitivity to PTX+cMab using CD-DST. Furthermore, we evaluated the mechanisms of the enhanced antitumor effect based on alterations in the expression of PTEN.

Materials and Methods

Materials. This study used 8 human OSCC cell lines: SAT, SAS, OSC-20, HSC-2, HSC-3 and HSC-4 (tongue squamous cell carcinoma); HO-1-u-1 and KON (oral floor squamous cell carcinoma). These OSCC cell lines were purchased from the National Institutes of Biomedical Innovation, Health and Nutrition, Japanese Collection of Research Bioresources (JRCB) Cell Bank and cultured in Dulbecco's modified eagle's medium (DMEM/F12: Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 10% fetal bovine serum (10% FBS: Life Technologies, Van Allen Way, CA, USA), 0.1% Minimum essential media (MEM) non-essential amino acid solution, 1% Penicillin–Streptomycin and 0.1% Fungizone (Life Technologies) in a 35 mm plastic Petri dish at 37˚C in an incubator with 95% humidity and 5% CO_2. The anticancer drugs used were cetuximab (Erbitux® Injection 100 mg/20 ml, Merck Biopharma, Tokyo, Japan) and paclitaxel (Paclitaxel Inj.® 100 mg/16.7 ml, Nippon Kayaku, Tokyo, Japan). Female BALB/c/nu/nu nude mice were housed under specific pathogen-free conditions (Charles River Laboratories, Tokyo, Japan). The animal experiments in this study were approved by the Animal Committee of The Nippon Dental University, School of Life Dentistry at Niigata, Japan (Approval No. 131).

Evaluation of sensitivity of OSCC cell lines to PTX, cMab, and PTX+cMab using CD-DST. Anticancer drug sensitivity tests were performed using CD-DST and Primaster® (Kurabo Industries Ltd., Osaka, Japan), a primary human cancer cell culture system kit developed from the method reported by Kobayashi *et al*. (13); the test was performed for each cell line as directed (Figure 1). In the test, solution A (Cellmatrix® Type CD), solution B (10-fold concentration of F-12 medium), and solution C (reconstituted buffer solution) were mixed at a ratio of 8:1:1, and cancer cells were adjusted to 1-5×10^5 cells/ml prior to being mixed. A total of 90 μl were added per well to a 6-well non-treated plate using a micropipette. The solution mixed with the OSCC cell lines were allowed to make a gel by incubating the plate at 37˚C for 1 h and cultured in DMEM/F-12 (DF) medium containing 10% FBS for 24 h. In accordance with the report from Kii *et al*. (12), cells were exposed to 0.1 μg/ml of PTX for 24 h and, in accordance with the report from Ryuki *et al*. (14), to 250 μg/ml of cMab for 144 h. The anticancer drugs were the removed by washing the plates with phosphate buffered saline (PBS); the media were replaced with serum-free medium and cells were cultured for an additional 6 days. Following this, the cells were stained with neutral red solution for 2 h and fixed in 10% neutral formalin solution for 40 min. After fixation, the cells were washed with water and dried to prepare study samples. The prepared samples were subjected to response evaluation by image analysis.

Response assessment via image analyses. The antitumor effect of the anticancer drugs was evaluated with the method reported by Koezuka *et al*. (15) using the Primage® image analyzer (Kurabo Industries Ltd., Osaka, Japan). Gray-scale images were captured with the image analyzer, and images other than those of cancer cells were separated and removed according to the differences in the degree of staining of the images. The growth of cancer cells was confirmed, and the antitumor effects were determined by measuring colony volume based on the images of cancer cells. The ratio of the volume in the group exposed to anticancer drugs (T: Treatment) to the volume in the control (C: control) group, was used to determine the T/C value, which is the ratio of the proliferation rate of tumor cells exposed to anticancer drugs (T) to that of tumor cells not exposed to anticancer drugs (C), and the results were classified as:

Table I. *Primer sequence and PCR condition.*

Gene name	Primer sequences, 5' to 3'	Tm (˚C)	Length (bp)	Accession number
PI3KCA	F: GGCCACTGTGGTTGAATTGGGA R: AGTGCACCTTTCAAGCCGCC	59	280	NM_006218
AKT	F: GGGCTCTGGACTCCCGTTTG R: CCCTGCTCCCCAGACTAGGA	62	94	NM_005163
PTEN	F: TGGGCCCTGTACCATCCCAAGT R: TGTGGCAACCACAGCCATCGT	60	242	NM_001304717
GAPDH	F: AAGGTCGGAGTCAACGGATTTG R: GCCATGGGTGGAATCATATTGG	55	150	NM_002046

high or low sensitivity using the respective criteria of 0<T/C ≤50% and 50%<T/C. Samples with a cell growth rate <0.8-fold were excluded from the evaluation.

Identification of KRAS gene mutations via the Loop-Hybrid morbidity shift assay (LH-MSA). Loop-Hybrid Mobility Shift Assay (LH-MSA, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used to detect the presence or absence of KRAS gene mutations in codons 12 and 13 in the eight OSCC cell lines. Genomic DNA was extracted according to the ISOGENOME protocol (Nippon gene, Tokyo, Japan). Polymerase chain reaction (PCR) amplification was performed at 94˚C for 2 min → (94˚C for 20 s, 56˚C for 20 s, and 72˚C for 20 s) for 36 cycles → 72˚C for 2 min. The PCR amplification products were mixed with 1 μl each of the LH probes, and LH reactions were performed at 94˚C for 2 min followed by incubation at 68˚C for 5 min, and finally 3 μl loading buffers were added to each reaction. The LH reaction products were electrophoresed (constant current: 20 mA, 80 min) on polyacrylamide gels. Staining was performed with SYBR® Green I Nucleic Acid Gel Staining for 15 min and confirmed with an ultraviolet (UV)-irradiation gel imaging apparatus (LAS-1000, FUJIFILM Holdings Corporation, Tokyo, Japan).

Quantitative comparison of AKT, PI3KCA, and PTEN expression via real-time PCR. PI3KCA, AKT, and PTEN mRNA expression levels were quantitatively compared after exposure to anticancer drugs using real-time PCR (Table I). The samples were categorized into the following four groups: Control (not exposed to anticancer drugs), PTX exposed, cMab exposed, and PTX+cMab exposed groups. All groups of OSCC cell lines were 3D cultured in collagen gel. The control was not exposed to anti-cancer drugs, whereas the other groups were exposed to PTX and/or cMab. Collagen gels were lysed in cell dispersing enzyme (EZ, Kurabo Industries Ltd., Osaka, Japan) and cells were harvested. Following cell harvesting, total RNA was extracted using the ISOGEN II protocol (Nippon gene, Tokyo, Japan). Real-time PCR was performed using the PrimeScript RT TM reagent kit with gDNA Eraser (Perfect Real Time, TKARA BIO Inc., Shiga, Japan) according to the manufacturers' protocol and the levels of expression of PI3KCA, AKT, and PTEN were quantified by the ΔΔCT method. These genes were compared for changes in the expression levels after exposure to anticancer drugs. In addition, *PTEN* expression was also compared between OSCC cell lines before exposure to anticancer drugs. Results were normalized by GAPDH expression and presented as fold changes compared to the results in the control group. The experiments were repeated six times.

Evaluation of changes in PTEN expression levels. Owing to the presence of significant changes in the *PTEN* gene expression levels based on real-time PCR results, a western blot analysis was used to confirm the PTEN activity of OSCC cell lines after exposure to anticancer drugs. Furthermore, comparison of *PTEN* expression after administration of PTX+cMab *in vivo* using nude mice was performed *via* real-time PCR.

Western blot analysis of the expression of *PTEN*: PTX and/or cMab treated OSCC cells were used for the analysis of PTEN expression. Whole cell lysates from control and treated cells were prepared with RIPA buffer (NACALAI TESQUE, Inc., Kyoto, Japan). Cell lysates containing were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels, and then transferred to a PVDF membrane. The membranes were blocked and then treated with the anti-PTEN antibody (Abcam, Cambridge, UK), anti-rabbit IgG HRP conjugate (Promega Corporation, Madison, WI, USA).

Comparison of PTEN expression levels *in vivo* in xenograft tumors: The OSCC cell lines (1×10^7) were diluted with 0.5 ml of Hank's solution and administered subcutaneously to the back of nude mice using a 23G Terumo syringe®; the 5 cell lines HSC-2, OSC-20, SAS, SAT, and HSC-4 produced engraftments. Anticancer drugs were administered according to the method reported by Kii *et al.* (12) and Harada *et al.* (16) through intraperitoneal injection with PTX (20 mg/kg/day, twice/week, 3 weeks) and/or cMab (20 mg/kg/day, twice/week, 3 weeks). Twenty-one days after the start of anticancer drug administration, nude mice were sacrificed *via* intraperitoneal administration of somnopentyl (pentobarbital sodium) at 200 mg/kg. Tumors were removed and homogenized using a BioMasher® III. After this, total RNA was extracted and quantitative comparisons of PTEN expression *in vivo* were performed using real-time PCR.

Statistical analyses. The experimental results are expressed as mean±SD. The significance of all real-time PCR data was determined by one-way ANOVA. The Holm test was used for multiple comparisons. The differences were considered statistically significant when $p<0.05$. The statistical analysis software used was the Bell Curve for Excel version 3.20 (Social Survey Research Information, Tokyo, Japan).

Results

Sensitivity of OSCC cell lines to PTX, cMab, and PTX+cMab exposure via CD-DST. CD-DST results for PTX alone, cMab alone, and PTX+cMab are presented. High

Table II. *Results of CD-DST method testing 8 cell lines at PTX (0.1 μg/ml), cMab (250 μg/ml) and PTX + cMab contact. Results of the CD-DST method (T/C values) T/C value of ≤50% was scored as high sensitivity, and that of >50% was scored as low sensitivity. Bold data indicate high sensitivity.*

Cell-line	HSC-2	HO-1-u-1	OSC-20	SAS	HSC-3	SAT	HSC-4	KON
Drug								
PTX	54.3	67.5	79.2	**38.1**	**28.9**	82.2	71.2	71.9
cMab	67.9	91.2	88.1	82.1	84.1	81.5	79.3	78.2
PTX+cMab	**25.2**	**33.5**	**36.2**	**19.2**	**17.3**	70.1	62.3	67.9

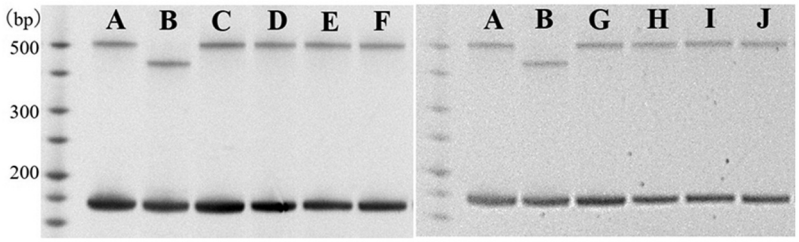

Figure 2. *No KRAS mutations were detected via Loop-Hybrid morbidity shift assay. A: Wild type, B: G12D mutation, C: SAS, D: SAT, E: HSC-2, F: HSC-3, G: HSC-4, H: HO-1-u-1, I: OSC-20, J: KON cells. PTX: Paclitaxel; cMab: cetuximab.*

sensitivity was defined as 0<T/C ≤ 50% and low sensitivity was defined as 50%<T/C. CD-DST results revealed that HSC-2, HO-1-u-1, and OSC-20 cells demonstrated low sensitivity to PTX or cMab alone and high sensitivity to PTX+cMab; SAS and HSC-3 cells demonstrated high sensitivity to PTX alone and PTX+cMab; and SAT, HSC-4, and KON cells demonstrated low sensitivity to PTX or cMab alone and PTX+cMab (Table II).

Presence or absence of KRAS mutations. LH-MSA was used to confirm the presence or absence of KRAS gene mutations. In all eight OSCC cell lines, no KRAS gene mutations were detected, and wild type KRAS expression was observed (Figure 2).

Quantitative comparison of PI3KCA, AKT, and PTEN expression via real-time PCR. PI3KCA expression was significantly increased with PTX alone, cMab alone, and PTX+cMab exposure ($p<0.01$) (Figure 3A). Although the expression of AKT increased in 7 of 8 cell lines after PTX exposure, and a significant increase was observed in 6 cell lines, no association with susceptibility was observed (Figure 3B). PTEN expression in the HSC-2, HO-1-u-1, and OSC-20 cell lines, which demonstrated high sensitivity only to PTX+cMab exposure, was not affected by PTX exposure but was increased with cMab exposure ($p<0.01$). In addition, there was a significant increase following PTX+cMab exposure compared to cMab alone ($p<0.01$). Although SAS and HSC-3 cell lines showed high sensitivity to PTX alone and PTX+cMab, the expression of PTEN did not change after exposure to PTX alone, whereas a significant increase was observed after exposure to PTX+cMab ($p<0.01$). Furthermore, the expression of PTEN in SAT, HSC-4, and KON cell lines, which demonstrated low sensitivity to PTX alone, cMab alone, and PTX+cMab, did not increase upon exposure to any of the anticancer treatments (Figure 3C). There were no significant differences in the PTEN expression between the OSCC cell lines before anticancer drug exposure (Figure 4).

Evaluation of changes in PTEN expression levels. Increased PTEN protein expression following treatment of cells with PTX+cMab exposure in vitro. To confirm whether PTEN protein expression is increased upon treatment with PTX+cMab, we examined its expression by western blotting. Cell lines with high sensitivity to PTX+cMab showed an increase in PTEN protein expression upon exposure to PTX+cMab. However, cell lines with low sensitivity to PTX+cMab showed no changes in the levels of PTEN protein expression after exposure to PTX+cMab (Figure 5).

Comparison of PTEN expression levels in vivo. As in the *in vitro* experiments, similar changes in PTEN expression were observed *in vivo*. Tumors developed from cell lines with high sensitivity to PTX+cMab showed a significant increase in PTEN expression upon exposure to PTX+cMab ($p<0.01$),

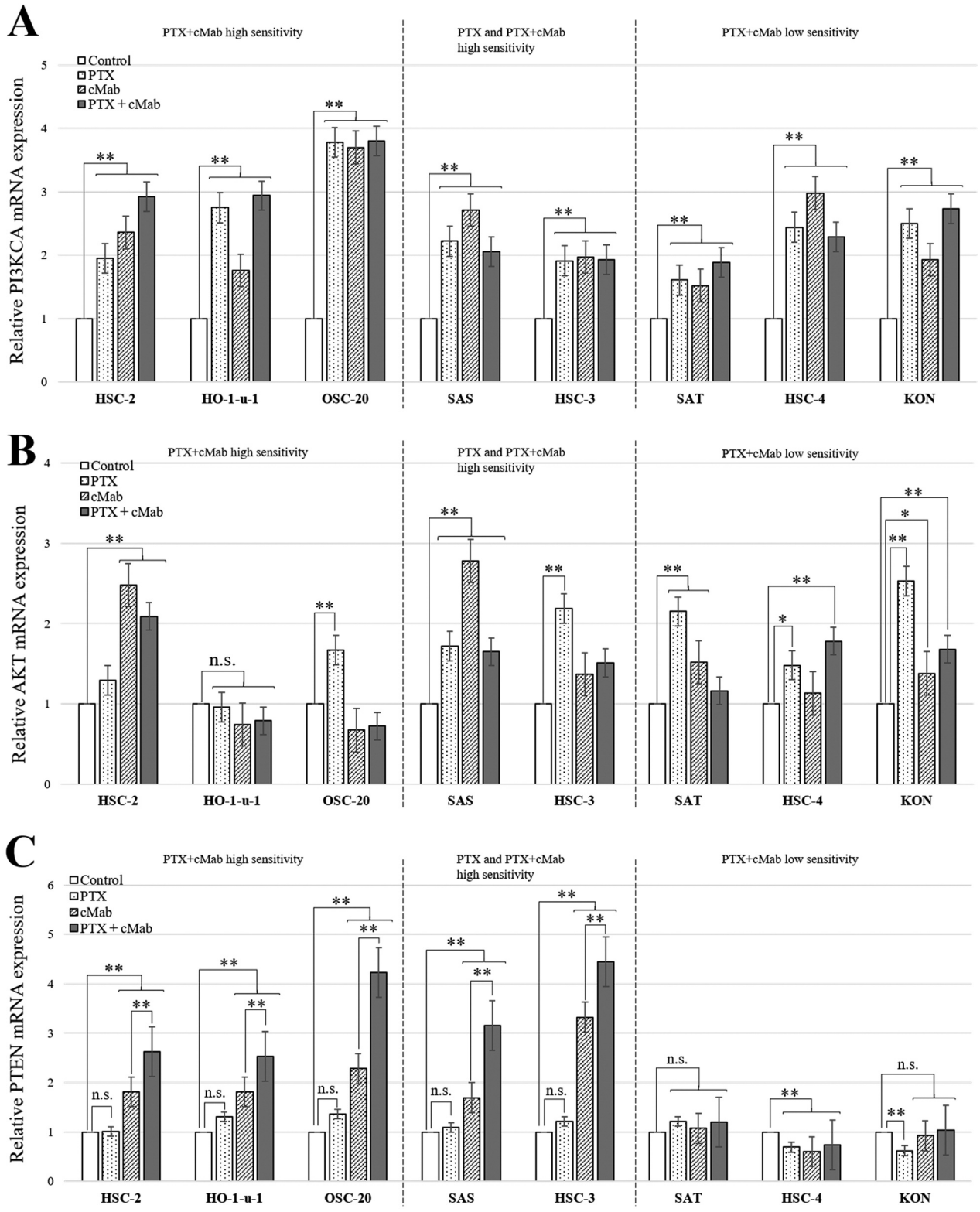

Figure 3. *PI3KCA, AKT and PTEN mRNA expression after exposure to anticancer drugs (in vitro). A) PI3KCA expression was significantly increased after exposure to paclitaxel (PTX), cetuximab (cMab), and PTX+cMab. B) AKT expression did not change after exposure to PTX, cMab, or PTX+cMab and no consistent trend was observed. C) PTEN expression was significantly increased after cell lines sensitive to PTX+cMab were exposed to PTX+cMab; however, this finding was not seen in cell lines not sensitive to PTX+cMab. *p<0.05, **p<0.01.*

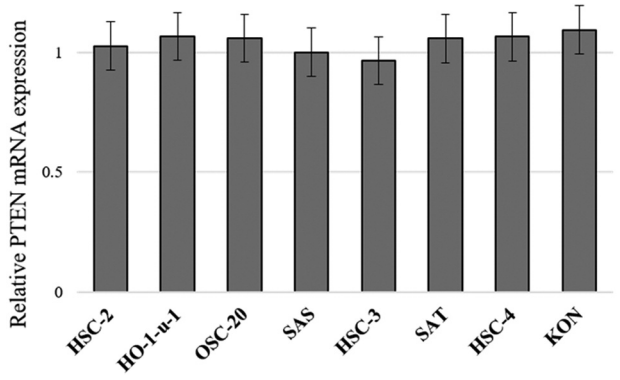

Figure 4. *PTEN mRNA expression before exposure to anticancer drug (in vitro). The levels of expression of PTEN in the eight oral squamous cell carcinoma cell lines before exposure to anticancer drugs were compared. There were no significant differences in PTEN expression before exposure to anticancer drugs.*

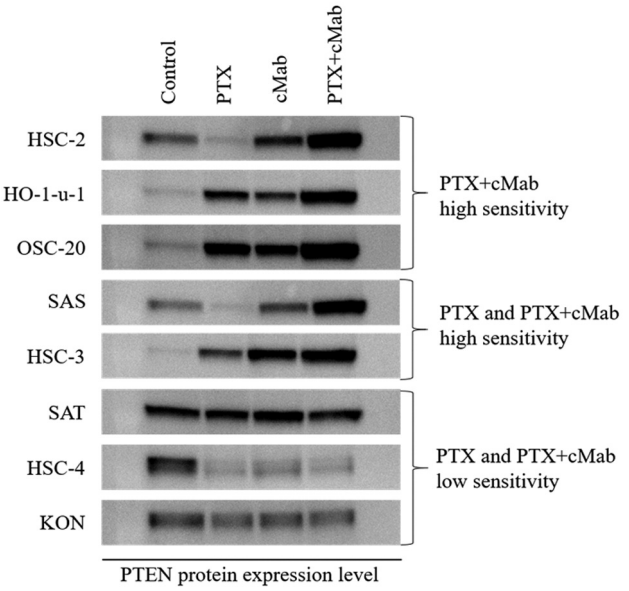

Figure 5. *Increased PTEN protein expression by paclitaxel (PTX) + cetuximab (cMab) exposure (in vitro). Expression of PTEN protein following treatment of cells with PTX, cMab or PTX+cMab was examined by western blotting. Cell lines with high sensitivity to PTX+cMab showed an increase in PTEN protein expression upon exposure to PTX+cMab.*

whereas tumors developed from cell lines with low sensitivity to PTX+cMab showed no changes in the levels of PTEN expression after exposure to PTX+cMab (Figure 6).

Discussion

For recurrent/metastatic head and neck cancer, the combination of the platinum drug cisplatin and cMab is used as first-line therapy and the benefits of this treatment have also been reported in a multicenter retrospective study conducted in Japan (17). However, this combination is not indicated for patients with serious renal disorders due to the renal impairment caused by cisplatin. Nivolumab was approved as a second-line therapy in Japan in 2017; however, the low response rate of 13.3% is an issue (18). Thus, PTX+cMab therapy, which is indicated for patients with renal impairment and associated with a high response rate, is indicated as a last-line treatment for recurrent/metastatic head and neck cancer. However, the add-on effect of cMab to PTX, the mechanism of enhancement of antitumor effects, and the cause of resistance remain unclear.

cMab reportedly competes with the effects of EGF on EGFR and blocks the downstream PI3K-AKT and RAS signaling pathways, resulting in antitumor effects (19). EGFR is reportedly expressed in approximately 90% of head and neck cancers (20). However, *KRAS* mutations, with frequencies of 43% reported for colorectal cancer (21) and of around 5% for head and neck cancer (3, 22, 23), result in cMab resistance in addition to the blockade of EGFR and the PI3K-AKT pathway (21). In this study, KRAS mutations were not observed. Since there is a low possibility of cMab resistance due to *KRAS* mutations, we focused on changes in *PI3KCA, AKT*, and *PTEN* genes downstream of EGFR.

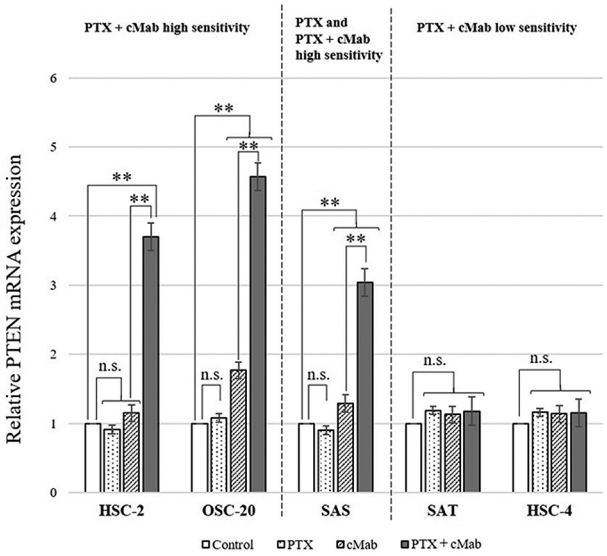

Figure 6. *PTEN mRNA expression after administration of anticancer drugs (in vivo). The levels of expression of PTEN in tumors were compared after paclitaxel (PTX), cetuximab (cMab), and PTX+cMab treatment in nude mice. The results were similar to those in vitro, i.e., tumors derived from cell lines sensitive to PTX+cMab showed a more marked increase in PTEN expression after exposure to PTX+cMab. **p<0.01.*

PI3K, AKT, and *PTEN* genes have been reported to be predictors of the therapeutic effects of cMab and PTX in various types of cancer (24, 25). However, none have been defined, as most of the studies have compared gene expression levels in monolayer-cultured cell lines or in cancer tissues before anticancer drug administration (7-9). Thus, we utilized 3D cultures and assessed the expression of *PI3KCA, AKT*, and *PTEN* genes in 3D cultures before and after exposure to cMab and PTX. CD-DST enables long-term 3D cultures that can simulate *in vivo* conditions and, furthermore, allows exposure to anticancer drugs at the same concentration as in the clinical settings (11, 12, 14). Ohnishi *et al*. reported that OSCC cell lines grown in monolayers were resistant to cMab, but became sensitive, formed aggregates, and showed activation of the PI3K-AKT pathway when grown as floating cultures (26). In other words, monolayer cultures may not represent the best model for the determination of relevant genetic changes. 3D cultures that can be reproduced *in vivo* were considered to be important for observing genetic changes in the PI3K-AKT pathway.

In this study, the expression of *PI3KCA* significantly increased with exposure to PTX or cMab regardless of the OSCC cell line or the sensitivity to anticancer drug ($p<0.01$). Tsuruo *et al*. reported that cancer cells showed increased expression of oncogenes in response to chemotherapy (25), suggesting that *PI3KCA* levels may increase as part of a negative feedback mechanism. AKT expression levels increased after exposure of some cell lines to anticancer drugs. Wu G *et al*. reported that PTX-resistant gastric cancer cell lines have higher levels of phosphorylated AKT (24). However, SAS and HSC-3, which demonstrated high sensitivity to PTX alone, showed an increase in AKT expression levels; the relationship with PTX resistance is not clear. Higher levels of phosphorylated AKT in head and neck squamous cell carcinoma (HNSCC) cell lines have also been reported to be involved in resistance to anti-EGFR agents (27). In this study, no association was found between AKT expression levels and the sensitivity to anticancer drugs.

In this study, no relationship between PI3KCA, AKT and the mechanism of enhanced antitumor effects of PTX+cMab was found; however, a certain tendency was observed for PTEN after exposure to anticancer drugs. PTEN has been reported to inhibit the PI3K-AKT pathway and cancer cell growth (28). In recurrent/metastatic head and neck cancer, PTEN expression is measured in tumor tissues before administration of anticancer drugs and its low expression has been associated with cMab resistance (7-9). In this study, although there were no differences in PTEN expression among the eight cell lines before exposure to anticancer drugs, a clear increase in PTEN expression was observed after exposure of sensitive cell lines to PTX+cMab. In contrast, in the cell lines that were not sensitive to PTX+cMab, PTEN expression did not increase after exposure to PTX+cMab, similar to results seen *in vivo*. Furthermore, western blotting showed a clear increase in PTEN protein levels after exposure of sensitive cell lines to PTX+cMab. It was found that PTEN mRNA and protein expression levels were increased and PTEN was activated in OSCC cell lines that were high sensitivity to PTX + cMab.

These results suggest that the expression of PTEN before exposure to anticancer drugs alone cannot be used to evaluate the potentiating effect of the combination treatment of cMab and PTX in OSCC; however, an increase in PTEN is clearly involved in the enhancement of antitumor effects by the addition of cMab to PTX and, if the factor that increases the expression of PTEN is identified, it may be a predictive biomarker of the effect.

In summary, increased *PTEN* expression and its activation may be involved in the enhancement of the antitumor effects of PTX after the addition of cMab. Future studies should investigate the underlying cause for the PTX+cMab-induced *PTEN* expression and its activation.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

KT and KS made substantial contributions to the conception and design of the study, as well as in the acquisition, analysis, and interpretation of the data. KS and AT were involved in the drafting and critical revision of the manuscript for important intellectual content.

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