# Role of high ubiquitin-conjugating enzyme E2 expression as a prognostic factor in nasopharyngeal carcinoma

WAN-CHEN KAO[1], SHIH-HAN HSU[2], CHIEN-LIANG LIN[1], CHENG-YAO LIN[1], SHANG-WEN CHEN[1], YAN-XUN CHEN[1], CHAO-HSUN CHEN[1], SUNG-WEI LEE[3], CHAO-JUNG TSAO[1], WEN-TSUNG HUANG[1], SHANG-HUNG CHEN[2,4] and SHENG-YEN HSIAO[1,5]

[1]Division of Hematology-Oncology, Department of Internal Medicine, Chi Mei Medical Center, Liouying, Tainan 736402; [2]National Institute of Cancer Research, National Health Research Institutes, Tainan 704016; [3]Department of Radiation Oncology, Chi Mei Medical Center, Liouying, Tainan 736402; [4]Department of Oncology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University; [5]Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan 701401, Taiwan, R.O.C.

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**Abstract.** The incidence of nasopharyngeal carcinoma (NPC) in Southeast Asia and Taiwan is high due to epidemiological factors. Cisplatin-based chemoradiotherapy is an important treatment strategy with excellent outcomes for patients with NPC. However, the outcomes for patients who are refractory to cisplatin-based therapy are poor. Methods for risk stratification of patients with NPC undergoing cisplatin-based chemoradiotherapy require to be investigated. A previous study indicated that ubiquitin-conjugating enzyme E2 B (UBE2B) was able to regulate alkylating drug sensitivity in NPC cells. In the present study, the clinical significance of UBE2B expression in patients with NPC was analyzed. Analysis of the two available NPC datasets containing the UBE2B expression profile (GSE12452 and GSE68799) was performed to evaluate the UBE2B expression levels in NPC tissues compared with nasopharyngeal mucosal epithelial tissues. Furthermore, immunohistochemical staining was performed using anti-UBE2B antibodies on samples from 124 patients with NPC who underwent cisplatin-based chemoradiotherapy. Disease-specific survival (DSS), distant metastatic-free survival (DMeFS) and local recurrence-free survival (LRFS) of patients with high and low UBE2B expression was analyzed. Furthermore, the associations between UBE2B expression and the biological behavior of NPC cells were investigated *in vitro*. Using public NPC datasets and *in vitro* studies, it was identified that UBE2B expression levels were increased in NPC tumor tissues compared with those in mucosal epithelial tissues. The cell proliferation ability was decreased in UBE2B-deficient NPC cells as compared with that in UBE2B-proficient cells. Immunohistochemical analysis of 124 NPC tissues from patients who underwent cisplatin-based chemoradiotherapy indicated that high UBE2B expression levels were associated with poor DSS, DMeFS and LRFS. Multivariate regression analysis of factors influencing survival also confirmed that high UBE2B expression levels were a statistically significant independent risk factor for poor clinical outcomes in terms of DSS [hazard ratio (HR), 1.955; 95% CI 1.164-3.282], DMeFS (HR, 2.141; 95% CI 1.206-3.801) and LRFS (HR, 2.557; 95 CI 1.313-4.981). *In vitro* analysis indicated that O6-methylguanine-DNA methyltransferase attenuated cisplatin sensitivity induced by knockdown of UBE2B in NPC cells. In conclusion, the present study demonstrated that high UBE2B expression is associated with poor clinical outcomes for patients with NPC treated with cisplatin-based chemoradiotherapy.

*Correspondence to:* Dr Shang-Hung Chen, National Institute of Cancer Research, National Health Research Institutes, 2F 367 Sheng-Li Road, North, Tainan 704016, Taiwan, R.O.C.
E-mail: bryanchen@nhri.edu.tw

Dr Sheng-Yen Hsiao, Division of Hematology-Oncology, Department of Internal Medicine, Chi Mei Medical Center, 201 Taikang, Liouying, Tainan 736402, Taiwan, R.O.C.
E-mail: seedvirt@gmail.com

*Abbreviations:* NPC, nasopharyngeal carcinoma; MGMT, O6-methylguanine-DNA methyltransferase; UBE2B, ubiquitin-conjugating E2 enzyme B; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DSS, disease-specific survival; DMeFS, distant metastasis-free survival; LRFS, local recurrence-free survival; RFS, recurrence-free survival; OS, overall survival

*Key words:* ubiquitin-conjugating enzyme E2 B, nasopharyngeal carcinoma, cisplatin, chemoradiotherapy

## Introduction

Nasopharyngeal carcinoma (NPC) is a rare cancer type that accounts for 0.7% of all cancer cases worldwide, according to 2018 estimates of cancer incidence by the International Agency for Research on Cancer (1). However, the incidence of NPC in Southeast Asia and Taiwan is much higher due to epidemiologic factors, such as Epstein-Barr virus infection, environmental

factors, host genetics and smoking (2,3). Cisplatin-based concurrent chemoradiotherapy is now the standard treatment for locoregionally advanced disease and contributes to good prognoses as follows: 5-year failure-free survival rates of >70% and 5-year overall survival (OS) rates of >80% (4). Compared with patients with other head and neck cancers, patients with NPC are much younger and achieve higher distant metastatic rates. For metastatic disease, cisplatin plus fluorouracil has been the first-line treatment of choice; however, outcomes have been disappointing, with a 40-60% response rate, leading to a median OS of 10-15 months (2). Certain patients with metastatic disease have poor response, particularly when previously exposed to cisplatin treatment. Furthermore, rare treatment choices may be considered for progressive disease refractory to cisplatin-based treatments. There is currently no targeted therapy available for patients with advanced NPC. Exploring biomarkers for predicting cisplatin resistance and determining the resistance mechanisms are important research goals for NPC.

Protein degradation via ubiquitination is an important mechanism of cellular metabolism, during which the ubiquitin-activating enzyme E1 cooperates with the ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3 to complete protein ubiquitination (5). Several literature studies have demonstrated that the intracellular ubiquitination system may be implicated in oncogenic process through cell cycle control, metabolic pathway regulation and DNA damage response (6,7). The DNA repair enzyme, O6-methylguanine-DNA methyltransferase (MGMT), modulates the cytotoxicity of several alkylating agents (such as cisplatin) in relation to the DNA alkylation of the O6-position of guanine. According to a previous study, the potential regulator of MGMT, ubiquitin-conjugating E2 enzyme B (UBE2B), has the strongest ability to bind to MGMT and regulate MGMT ubiquitination mediated by alkylating agents (8). Furthermore, a previous study by our group demonstrated that MGMT repairs platinum-DNA adducts and MGMT proficiency contributes to the poor prognosis of patients with NPC (9). These results suggest a role of UBE2B expression in predicting survival outcomes for patients with NPC receiving cisplatin-based therapy.

The present study aimed to determine UBE2B expression in the malignant tissue of patients with NPC and evaluate UBE2B as a risk factor contributing to poor treatment outcomes of cisplatin-based chemoradiotherapy. First, UBE2B expression was compared between normal nasopharyngeal mucosa and NPC tissues by reappraising two datasets from a public database. The prognostic value of UBE2B was further examined in a Taiwanese NPC cohort comprising 124 patients receiving cisplatin-based chemoradiotherapy.

## Materials and methods

*Analysis of published NPC datasets*. UBE2B expression levels were analyzed in clinical NPC specimens and compared with nasopharynx mucosa tissue by searching for profiling datasets of gene expression in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). A total of two gene expression datasets satisfying these requirements, GSE12452 (Affymetrix-GPL570 platform) and GSE68799 (GPL11154 Illumina HiSeq 2000 platform), were reappraised for comparison (10). Regarding the microarray data of GSE12452, the preprocessed gene expression data were downloaded, whereas for the RNA-sequencing data of GSE68799, fragments per kilobase per million mapped reads were downloaded. All probe sets were analyzed without pre-selection or filtering. The UBE2B expression levels in NPC tissues were compared with those in nasopharynx tissues using the Wilcoxon rank-sum test. When multiple probe sets for the same gene symbol exhibited statistical significance, the probe values with the highest statistical difference between mucosa and tumor tissues were presented as UBE2B expression values.

*Cell culture*. The human NPC cell line TW01 and a human oral epithelial cell line (Dysplastic Oral Keratinocyte; DOK) used in the present study were obtained from the cell bank of the Taiwanese National Institute of Cancer Research, National Health Research Institutes. NPC cells were authenticated by the Authentication Services of Topgen Biotechnology. TW01 cells were incubated with α-MEM medium (Gibco; Thermo Fisher Scientific, Inc.) (8). DOK cells were routinely cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc.). All the culture media were supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimycotic solution (GeneDireX). All cells were cultured at 37˚C in a humidified incubator with 5% $CO_2$.

*Antibodies and reagents*. The rabbit polyclonal antibody against UBE2B (cat. no. GTX100416; 1:1,000 dilution) and rabbit monoclonal antibody against β-actin (cat. no. GTX109639; 1:10,000 dilution) were purchased from GeneTex. The mouse monoclonal antibody against MGMT (cat. no. MGMT2015#5; 1:5,000 dilution) was obtained from LTK BioLaboratories. The horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-2004 and sc-2005; 1:10,000 dilution) and the rabbit polyclonal antibodies against enhanced green fluorescence protein (EGFP) (cat. no. sc-8334; 1:1,000 dilution) were obtained from Santa Cruz Biotechnology, Inc. Other experimental chemicals were described in a previous report (9).

*Western blot analysis*. The detailed experimental conditions of western blot analysis were as previously described (8). In brief, protein was quantitated by a protein assay kit (cat. no. 5000006; Bio-Rad Laboratories, Inc.). Equal amounts of protein (30 $\mu$g) from the cell extract prepared with RIPA lysis buffer (Merck Millipore) were separated through 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon®-P; Merck Millipore). After blocking with 5% non-fat milk (Fonterra) at ambient temperature for 1 h, the membranes were probed with primary antibodies against UBE2B, MGMT and actin at 4˚C overnight. The membrane was then hybridized with horseradish peroxidase-conjugated secondary antibody at ambient temperature for 1 h. The immunoreactivities of the membranes were detected using the Western Lightning™ Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer, Inc.). The protein levels were measured using densitometric analysis with ImageJ 1.53 (National Institutes of Health) and normalized to actin signals.

*Small interfering (si)RNA transfection*. For UBE2B silencing, an siRNA technique was used as described previously (8). In brief, NPC cells in the exponential growth phase were transfected with control siRNA (cat. no. 12935146; Thermo Fisher Scientific, Inc.) or siUBE2B (cat. no. 4390824; Thermo Fisher Scientific, Inc.) using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After transfection for 24 h, the cells were subjected to pertinent experiments.

*Clonogenic assay*. After NPC cells were seeded at $5x10^4$ cells/well in 6-well plates overnight, the cells were transfected with control siRNA or siUBE2B for 24 h. The cells were then reseeded at 1,000 cells/well into 6-well plates and allowed to grow for 7-14 days, dependent on the growth rates of individual NPC cells. Following the application of methylene blue staining, the numbers of colonies formed, which consisted of at least 50 tumor cells, were manually recorded.

*Patients and tumor specimens*. All formalin-fixed and paraffin-embedded NPC tissues were approved for the present study by the institutional review board of Chi-Mei Medical Center in Taiwan (IRB10710-L01). The specimens were obtained from the BioBank of Chi-Mei Medical Center (Tainan, Taiwan) and were anonymously processed for research purposes that excluded the identification of the participants' personal information. Informed consent was obtained from all subjects involved in the study. Available paraffin-embedded tissue blocks were retrieved from 124 patients with NPC diagnosed between January 1993 and December 2002. No signs or events of distant metastases were identified at the initial diagnosis. A total of two pathologists reappraised the histological subtypes according to the criteria of the current World Health Organization (WHO) classification (11). The tumor staging of these samples was re-examined using the 7th American Joint Committee on Cancer system (12).

*Immunohistochemistry and assessment of UBE2B expression*. Tissue sections (3 $\mu$m) were obtained from paraffin-embedded blocks, as previously described (13). The slides were deparaffinized, rehydrated with ethanol and heated in a 10 mM citrate buffer (pH 6.0) by microwave for 7 min to retrieve antigens. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min at ambient temperature. The slides were then washed with tris-buffered saline and incubated with a primary rabbit polyclonal antibody against UBE2B (1:100 dilution; GeneTex, Inc.) for 1 h. A ChemMate EnVision kit (DAKO; Agilent Technologies, Inc.) was applied to detect the primary antibody. Tissue sections incubated with rabbit IgG in place of primary antibody served as negative controls. A total of two pathologists who were blinded to this study examined the UBE2B expression and scored the intensity and distribution with a multiheaded microscope to reach a consensus on the histology (H)-score using the following equation: H-score=$\Sigma$Pi (i+1) where i represents the intensity of stained tumor cells, and Pi is the percentage of stained tumor cells, ranging from 0 to 100%. Tumors with H-scores higher than the median value for all examined samples were classified as having high UBE2B expression levels.

*Treatment and follow-up*. All 124 patients with NPC completed the radiotherapy course, including a daily fractionation of 180-200 cGy and 5 fractions weekly to achieve a total dose of no less than 7,000 cGy. Patients with stage II-IV NPC also underwent at least 3 cycles of cisplatin-based chemotherapy, with all treatments performed according to the published protocol (14). The patients' responses were classified according to the WHO criteria (15). In total, there were 110 complete and 7 partial tumor regressions.

*Plasmid construction*. The pEGFPc1-MGMT plasmid was constructed as described previously (8). In brief, human MGMT cDNA was amplified from the previously constructed pTRE2hyg-MGMT plasmid (Clontech; Takara Bio, USA), which was kindly gifted by Professor Jang-Yang Chang (Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Miaoli, Taiwan) (16). After performing PCR, the amplified products of MGMT cDNA (0.624 kb) were then cloned into the pEGFPc1 vector (Clontech; Takara Bio USA) (8,16). The clones were verified by sequencing with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an AB 3130xl Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

*Cell viability assay*. After seeding $1x10^4$ cells/well into 24-well plates overnight, NPC cells were transfected with 20 nM control siRNA, siUBE2B or siUBE2B with 0.5 $\mu$g/well pEGFPc1-MGMT for an additional 24 h. To evaluate drug sensitivity, NPC cells were subsequently exposed to indicated concentrations of cisplatin (0, 0.125, 0.25, 0.5 and 1 $\mu$M cisplatin) for 3 growth generations. After the cells were fixed and stained using methylene blue, cisplatin cytotoxicity was measured as described previously (8). In brief, the survival rates of NPC cells with the indicated treatments were calculated from the absorbance (A) of methylene blue-stained cells as follows: Survival rate=$(A_{\text{tested cells}}-A_{\text{background controls}})/(A_{\text{matched control cells}}-A_{\text{background controls}})$ x100%. The $IC_{50}$ values (50% inhibition of cell viability) were determined based on the dose-response curves.

*Statistical analysis*. SPSS 16.0 statistics software (SPSS, Inc.) was employed for statistical analysis. To compare the mean or median of each group with that of the control group, the Mann-Whitney U-test was used. Data from the cell viability assay and clonogenic assay were examined using analysis of variance (ANOVA). If statistical significance of a treatment effect was obtained using ANOVA, Tukey's multiple-comparisons test was applied to determine the difference between treatment groups. To evaluate the associations between UBE2B expression and various clinicopathological variables, the $\chi^2$ test or Fisher's exact test was performed, depending on the group size. For the patient cohort, the following three endpoints were calculated: Disease-specific survival (DSS), distant metastasis-free survival (DMeFS), and local recurrence-free survival (LRFS), which were determined from the start date of the radiotherapy to the onset of an event. The Kaplan-Meier method with the log-rank test was applied to compare survival between groups. For multivariate analysis of factors influencing survival, linear logistic regression with

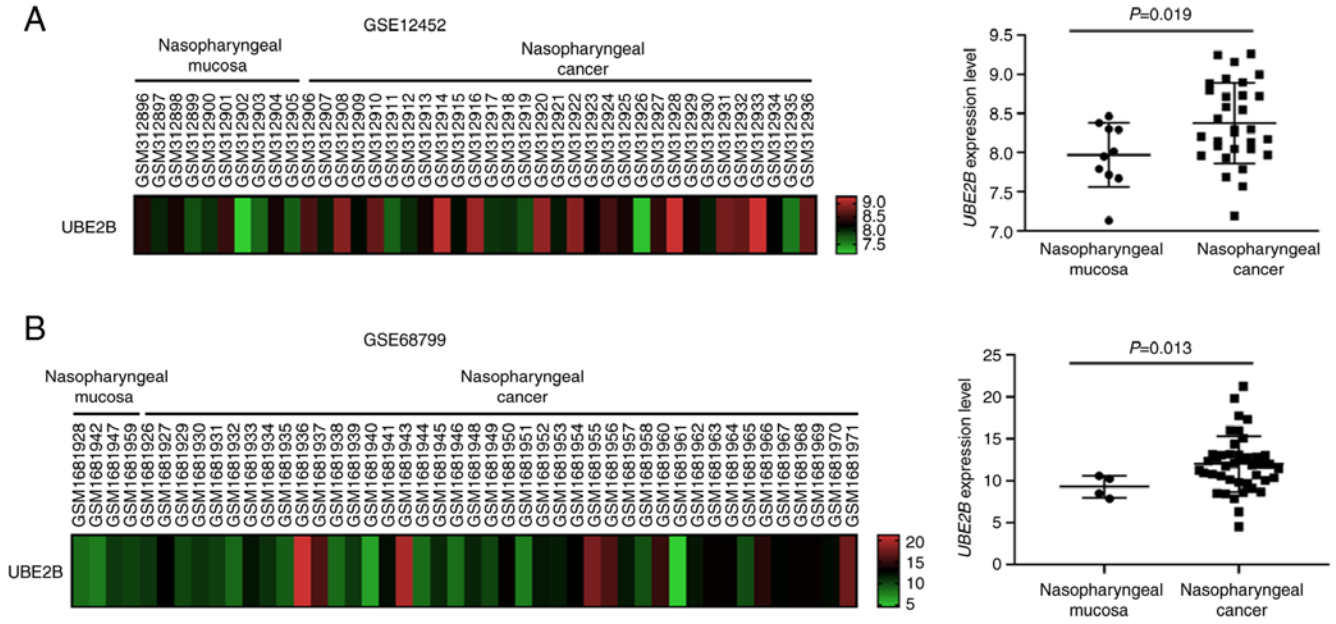


Figure 1. Increased expression levels of UBE2B in NPC tissues. Analysis of the public datasets (A) GSE12452 and (B) GSE68799 indicated increased expression levels of UBE2B in NPC compared to normal nasopharynx tissues. The heatmaps (left panel) present UBE2B transcription levels in each tissue sample and the scatter plots (right panel) indicate gene expression compared between normal nasopharyngeal mucosa and NPC tissues. The GSE12452 dataset contained mRNA signals from 10 non-NPC and 31 NPC tissues and the GSE68799 dataset contained 4 non-NPC and 42 NPC tissues. NPC, nasopharyngeal carcinoma; UBE2B, ubiquitin-conjugating enzyme E2 B.

the Cox proportional hazards model was applied. Two-sided tests were used for all analyses and P<0.05 was considered to indicate statistical significance.

## Results

*High UBE2B expression is frequently observed in NPC tissues.* A total of two public datasets (GSE12452 and GSE68799) were analyzed in order to explore the importance of UBE2B expression in the carcinogenesis of NPC. The GSE12452 dataset provided mRNA expression profiling from 31 NPC and 10 normal healthy nasopharyngeal tissue specimens. The subject information regarding age and sex was not available for the GSE12452 dataset. Among the GSE68799 dataset, which is a collection of mRNA profiles of 42 patients with NPC and 4 non-NPC tissues, the number of males was 35 and the mean age was 54 years. The clinicopathological data of these two datasets downloaded from the GSE database were summarized in Tables SI and SII. By examining mRNA expression levels in NPC tissues (n=31 and 42 for GSE12452 and GSE68799, respectively) and nontumor nasopharyngeal tissues (n=10 and 4 for GSE12452 and GSE68799, respectively) in these datasets, it was determined that UBE2B expression levels were increased in cancer tissues. As presented in Fig. 1, UBE2B expression levels in NPC tissues were significantly higher than those in nontumor nasopharyngeal tissues (P=0.019 for GSE12452; Fig. 1A; P=0.013 for GSE68799; Fig. 1B). To validate whether UBE2B is involved in NPC carcinogenesis, UBE2B expression levels were also compared between non-cancerous epithelial cells (DOK cells) and NPC cells (TW01 cells). As presented in Fig. 2A and B, UBE2B levels in TW01 cells were increased by 2.7-fold compared with those in DOK cells. Furthermore, UBE2B-deficient NPC cells were established by using the siRNA technique (Fig. 2C). The results of the clonogenic assay demonstrated that the number of colonies formed was decreased by 67% in UBE2B-deficient TW01 cells, as compared with that in their control counterpart (Fig. 2D and E). All of these results suggested that UBE2B is involved in the carcinogenesis of NPC.

*High UBE2B expression correlates with poor clinical outcome for patients with NPC receiving cisplatin-based concurrent chemoradiotherapy.* To validate UBE2B as a biomarker for predicting clinical outcome for patients with NPC, immunohistochemical staining of NPC tissues using anti-UBE2B monoclonal antibodies was employed (Fig. 3A and B). Based on the nuclear and cytoplasmic staining intensity and percentage of the tumor cells analyzed by two pathologists to determine the H-score of NPC tumor tissues, patients were stratified into high and low UBE2B expression groups. The median H-score was used as the cut-off point for distinguishing high and low UBE2B expression in NPC tumor tissues. The UBE2B expression was analyzed in tumor tissues from patients treated with cisplatin-based chemoradiotherapy at the Chi-Mei Medical Center (Tainan, Taiwan) from January 1993 to December 2002. Among the 124 collected NPC cases, the number of males was 95 and the mean age was 48.6 years. The H-scores in the low expression group were significantly lower than those in the high expression group according to the Mann-Whitney U-test (median H-score=200 and 300 for low- and high-expression group, respectively; P<0.001). In this patient population, the clinicopathological variables, such as sex, age, primary tumor status, nodal status, clinical stage or histological grade, exhibited no difference between the high and low UBE2B expression populations (Table I). However, the patient group with high UBE2B expression had

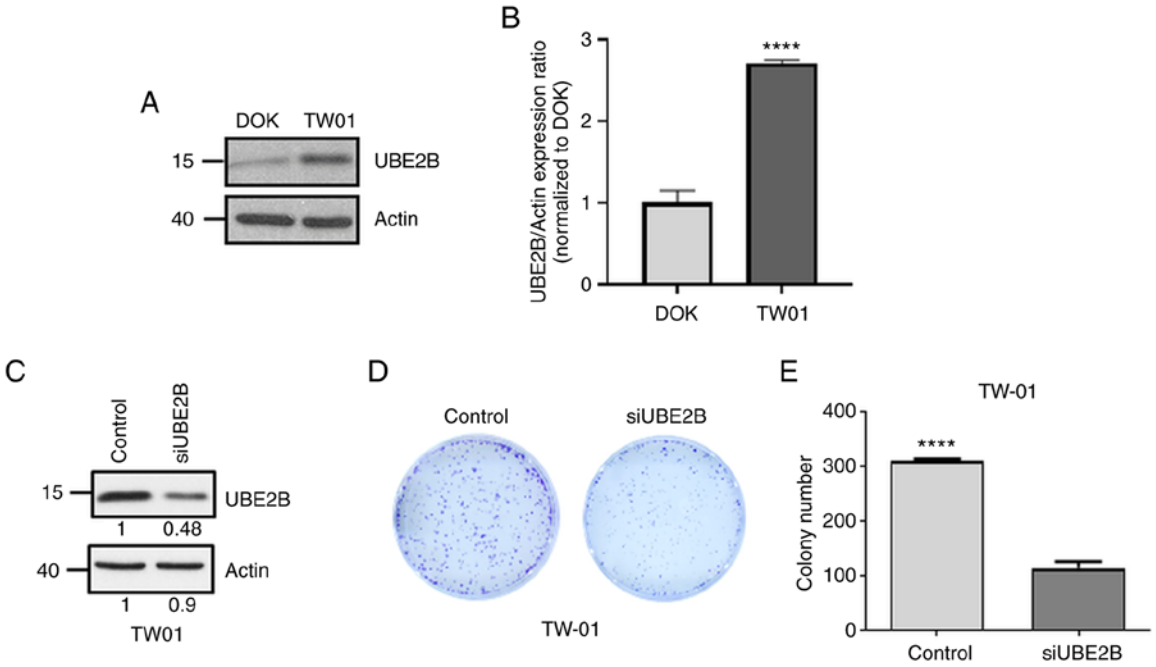


Figure 2. UBE2B has an important role in the carcinogenesis of NPC cells. (A) Western blot analysis demonstrated that UBE2B expression levels were higher in TW01 cells than those in DOK cells. (B) Bar graphs indicated a higher UBE2B/actin ratio in TW01 cells compared with that in DOK cells. (C) Expression levels of UBE2B in NPC cells treated with control or UBE2B-targeting siRNA were determined using western blot analysis. (D) By using methylene blue staining, the numbers of colonies formed, which consist of at least 50 tumor cells, were manually recorded and compared. (E) Bar graphs indicated decreased numbers of formed colonies in UBE2B-deficient NPC cells as compared with UBE2B-proficient cells. At least three independent experiments were performed and values were expressed as the mean ± standard deviation. ****P<0.01 for NPC cells vs. DOK cells and siUBE2B vs. control. Fold changes in protein levels listed under each blot were normalized to the levels of the control counterparts and analyzed by using ImageJ densitometry analysis. NPC, nasopharyngeal carcinoma; UBE2B, ubiquitin-conjugating enzyme E2 B; siRNA, small interfering RNA.

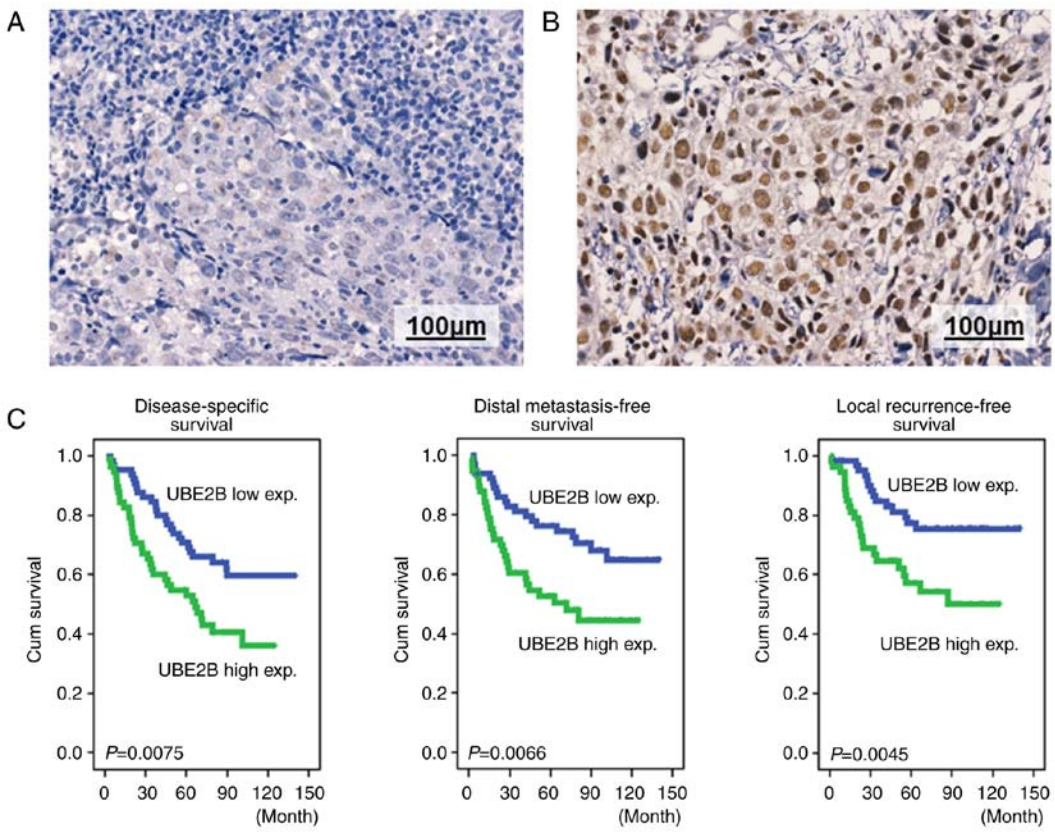


Figure 3. UBE2B expression levels are a prognostic marker for patients with NPC receiving cisplatin-based chemoradiotherapy. Immunohistochemical analysis of UBE2B indicated nuclear and cytoplasmic staining in representative NPC cases with (A) low (H-score=125) and (B) high (H-score=375) expression (scale bars, 100 $\mu$m). (C) Survival analysis revealed that high expression of UBE2B was a prognostic marker for poor disease-specific survival, distal metastasis-free survival and local recurrence-free survival. NPC, nasopharyngeal carcinoma; UBE2B, ubiquitin-conjugating enzyme E2 B; Cum, cumulative.

Table I. Associations between UBE2B expression levels and important clinicopathological variables.

| Parameter | UBE2B expression, n | | P-value |
|---|---|---|---|
| | Low | High | |
| Sex | | | 0.506 |
| Male | 49 | 46 | |
| Female | 17 | 12 | |
| Age, years | | | 0.416 |
| <60 | 54 | 44 | |
| ≥60 | 12 | 14 | |
| Primary tumor (T) | | | 0.363 |
| T1-T2 | 45 | 35 | |
| T3-T4 | 21 | 23 | |
| Nodal status (N) | | | 0.666 |
| N0-N1 | 31 | 25 | |
| N2-N3 | 35 | 33 | |
| Stage | | | 0.486 |
| I-II | 22 | 16 | |
| III-IV | 44 | 42 | |
| Histological grade | | | 0.346 |
| Keratinizing | 4 | 1 | |
| Non-keratinizing | 26 | 28 | |
| Undifferentiated | 36 | 29 | |

UBE2B, ubiquitin-conjugating enzyme E2 B.

poorer DSS, DMeFS and LFRS (Fig. 3C and Table II). The log-rank analysis also demonstrated that advanced primary tumor status, nodal status and clinical stage were associated with poor DSS, DMeFS and LRFS. Combined with UBE2B expression status and clinicopathological variables, the multivariate survival analysis revealed that the clinical stage remained an independent prognostic factor for DSS [hazard ratio (HR)=2.743, P=0.004], DMeFS (HR=2.564, P=0.011) and LFRS (HR=3.884, P=0.005), as presented in Table III. Furthermore, high UBE2B expression was also associated with shorter DSS (HR=1.955, P=0.011), DMeFS (HR=2.141, P=0.009) and LRFS (HR=2.557, P=0.006).

*MGMT is involved in UBE2B-mediated cisplatin sensitivity.* To evaluate the role of UBE2B in cisplatin sensitivity, the siRNA technique was used to silence UBE2B expression in NPC cells. When NPC cells were treated with 0.5 $\mu$M cisplatin, the results of cell viability assays indicated that cell cytotoxicity was increased by 26% in UBE2B-depleted TW01 cells as compared to control cells (Fig. 4). In a clonogenic assay, the $IC_{50}$ value of cisplatin was decreased by 64% in UBE2B-depleted TW01 cells as compared to control cells (Table IV). Furthermore, western blot analysis indicated cisplatin treatment (10 $\mu$M for 8 h) reduced UBE2B expression levels by 14% in TW01 cells as compared with the control cells (Fig. S1). These results suggested that UBE2B expression modulates cisplatin sensitivity in NPC cells. Previous studies have reported that UBE2B is able to regulate MGMT activity, which is also a determinant of cisplatin sensitivity in NPC cells (8,9). To examine whether MGMT is involved in UBE2B-mediated cisplatin sensitivity, NPC cells were transfected with MGMT-overexpression plasmids (Fig. S2). As presented in Fig. 4 and Table IV, MGMT expression attenuated cisplatin cytotoxicity in UBE2B-depleted NPC cells. The $IC_{50}$ value of cisplatin was increased by 100% in UBE2B-depleted TW01 cell cells transfected with MGMT-overexpression plasmid as compared with UBE2B-depleted NPC cells. These results indicate the participation of MGMT in UBE2B-mediated cisplatin sensitivity of NPC cells.

## Discussion

The global incidence and mortality rate of NPC have gradually declined, particularly in endemic areas (17,18), due to improvements in multidisciplinary cooperation and lifestyle changes (2). Patients with early-stage NPC are treated with radiotherapy alone with curative intent, while locoregionally advanced diseases require chemotherapy combined with radiotherapy. However, certain patients are at advanced stages at initial presentation and exhibit poor response to standard cisplatin-based chemoradiotherapy. Exploring the risk stratification for poor responders and developing novel targets for NPC treatment may help to improve survival outcomes for these patients. Several studies have examined the genetic landscape of NPC and explored the potential targetable pathways to improve treatment in NPC, including NF-κB pathway dysregulation, cell-cycle alteration and the PI3K/MAPK signaling pathway (2,19-21). Numerous theories have discussed drug resistance in NPC treatment, such as the targeting p53-MDM2 interaction for NPC treatment (22), stem cell-like properties induced by the leucyl-tRNA synthetase-mitotic arrest deficient 1 like 1 fusion gene through the far upstream element binding protein 1/c-Myc axis (23), cisplatin resistance contributed by higher expression levels of never in mitosis associate related kinase 2 (24), and transforming growth factor beta signaling pathway alteration induced by microRNA-449b (25). However, these theoretical mechanisms of drug resistance have not been established and applied in clinical practice.

Protein ubiquitination and degradation via the proteasome, and localization or interaction with other proteins, have an important role in oncogenic signaling (6). A crucial step in ubiquitination is the cooperation with the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2 and the ubiquitin ligase E3. Several cellular processes implicated in cancer progression, such as cell-cycle progression, receptor down-regulation, apoptosis and gene transcription, are regulated by the ubiquitination process (7). Therefore, the dysregulated ubiquitination processes may lead to mechanisms of anticancer agent resistance, such as E3 ubiquitin ligases contributing to the resistance mechanism by modulating pluripotent cancer stem cells (26). A previous study also revealed that E3-ligase Skp2 is able to regulate the cancer stem cell pool and predict poor prognosis in NPC (27).

The ubiquitination process conducted by UBE2B, which is highly conserved in eukaryotic cells, has several important cellular mechanisms, such as facilitating DNA methyltransferase DNMT3a ubiquitination and leading to gene promotor

Table II. Univariate log-rank analyses.

| Parameter | No. of cases | DSS | | DMeFS | | LRFS | |
|---|---|---|---|---|---|---|---|
| | | No. of events | P-value | No. of events | P-value | No. of events | P-value |
| Sex | | | | | | | |
| Male | 95 | 45 | 0.7870 | 38 | 0.6128 | 30 | 0.3240 |
| Female | 29 | 14 | | 11 | | 7 | |
| Age, years | | | | | | | |
| <60 | 98 | 48 | 0.8600 | 42 | 0.3091 | 29 | 0.8206 |
| ≥60 | 26 | 11 | | 7 | | 8 | |
| Primary tumor (T) | | | | | | | |
| T1-T2 | 80 | 32 | 0.0289 | 25 | 0.0085 | 19 | 0.0180 |
| T3-T4 | 44 | 27 | | 24 | | 18 | |
| Nodal status (N) | | | | | | | |
| N0-N1 | 56 | 18 | 0.0008 | 17 | 0.0132 | 12 | 0.0160 |
| N2-N3 | 68 | 41 | | 32 | | 25 | |
| Stage | | | | | | | |
| I-II | 38 | 10 | 0.0020 | 9 | 0.0072 | 5 | 0.0026 |
| III-IV | 86 | 49 | | 40 | | 32 | |
| Histological grade | | | | | | | |
| Keratinizing/non-keratinizing | 47 | 20 | 0.1980 | 17 | 0.2753 | 15 | 0.9521 |
| Undifferentiated | 77 | 39 | | 32 | | 22 | |
| UBE2B expression[a] | | | | | | | |
| Low | 66 | 25 | 0.0075 | 20 | 0.0066 | 9 | 0.0045 |
| High | 58 | 34 | | 29 | | 28 | |

[a]The median expression was used as the cut-off. DSS, disease-specific survival; DMeFS, distal metastasis-free Survival; LRFS, local recurrence-free survival; UBE2B, ubiquitin-conjugating enzyme E2 B.

Table III. Multivariate survival analyses.

| Parameter | DSS | | | DMeFS | | | LRFS | | |
|---|---|---|---|---|---|---|---|---|---|
| | HR | 95% CI | P-value | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Stage | | | | | | | | | |
| I-II | 1 | - | 0.004 | 1 | - | 0.011 | 1 | - | 0.005 |
| III-IV | 2.743 | 1.387-5.422 | | 2.564 | 1.241-5.294 | | 3.884 | 1.510-9.990 | |
| UBE2B expression[a] | | | | | | | | | |
| Low | 1 | - | 0.011 | 1 | - | 0.009 | 1 | - | 0.006 |
| High | 1.955 | 1.164-3.282 | | 2.141 | 1.206-3.801 | | 2.557 | 1.313-4.981 | |

[a]The median expression was used as the cut-off. DSS, disease-specific survival; DMeFS, distal metastasis-free Survival; LRFS, local recurrence-free survival; HR, hazard ratio; CI, confidence interval; UBE2B, ubiquitin-conjugating enzyme E2 B.

demethylation (28). In cancer cells, MGMT is a DNA repair enzyme that is able to disrupt the cytotoxicity of alkylating agents. The results of a previous study by our group demonstrated that UBE2B cooperates with RAD18 to ubiquitinate MGMT, which is then degraded by the proteasome (8). Immunofluorescence studies demonstrated the co-localization of MGMT and UBE2B with a greater entry into nuclei in 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-treated NPC cancer cells, supporting the interaction hypothesis. Of note, a previous study by our group suggested that UBE2B downregulation reduced MGMT ubiquitination but led to increased BCNU cytotoxicity (8). The deactivated MGMT protein, caused by BCNU or established by MGMT mutant mimics, was accumulated in cancer cells through UBE2B-mediated

Table IV. $IC_{50}$ values of cisplatin in cells transfected with scrambles ctrl siRNA, siUBE2B or siUBE2B plus pEGFPc1-MGMT ($\mu$M).

| Cell type | Ctrl | 2BKD | 2BKD + MGMT |
|---|---|---|---|
| TW01 | 1.1±0.1 | 0.4±0.1[a] | 0.8±0.1[b] |

Values are expressed as the mean ± standard deviation. [a]Ctrl vs. 2BKD (P<0.0001); [b]2BKD vs. 2BKD+MGMT (P<0.0001). Ctrl, cells transfected with scrambled siRNA; 2BKD, cells transfected with siUBE2B; 2BKD + MGMT, cells transfected with siUBE2B plus pEGFPc1-MGMT. UBE2B, ubiquitin-conjugating enzyme E2 B; siRNA, small interfering RNA; MGMT, O6-methylguanine-DNA methyltransferase; pEGFP, plasmid expressing enhanced green fluorescence protein.

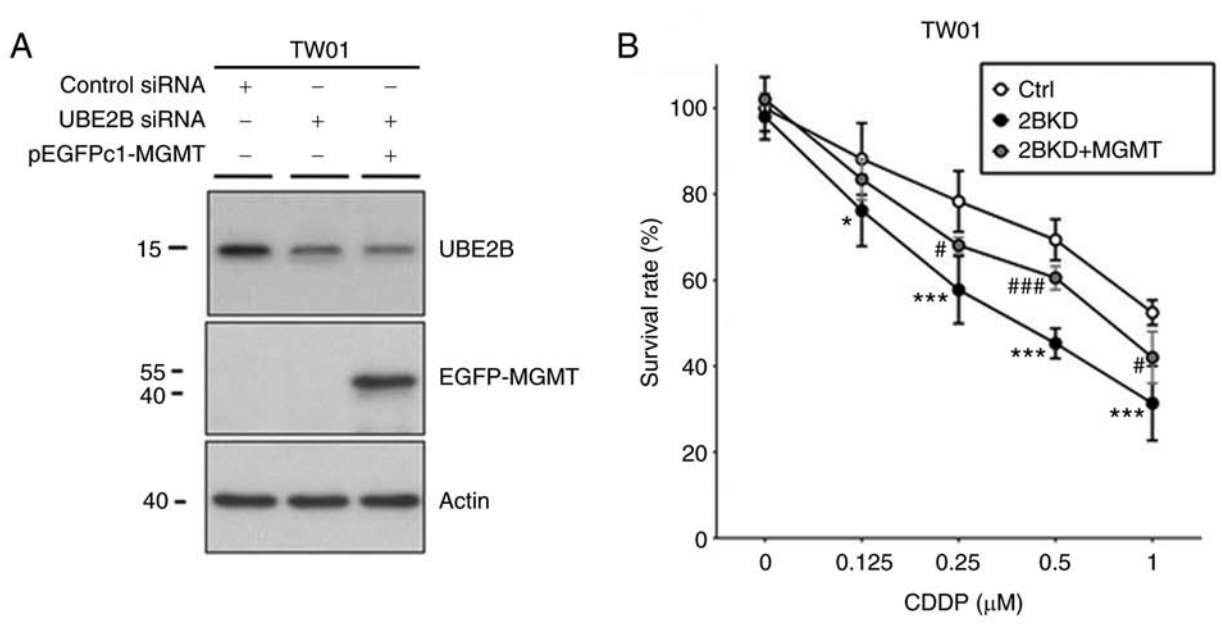


Figure 4. UBE2B modulates cisplatin cytotoxicity in nasopharyngeal carcinoma cells by targeting MGMT expression. (A) Western blot analysis demonstrated UBE2B and MGMT expression in TW01 cells with distinctive siRNA or plasmid transfection. (B) Cell viability assays were performed with TW01 cells to analyze the role of UBE2B and MGMT in cisplatin-induced cell death by using methylene blue staining. At least three independent experiments were performed. Cell survival results were presented as the mean ± standard deviation and compared using analysis of variance with Tukey's post-hoc test. *P<0.01, ***P<0.0001, 2BKD group vs. control group; #P<0.01, ###P<0.0001, 2BKD + MGMT group vs. 2BKD group. Ctrl, cells transfected with scrambled siRNA; 2BKD, cells transfected with siUBE2B; 2BKD + MGMT, cells transfected with siUBE2B plus pEGFPc1-MGMT. UBE2B, ubiquitin-conjugating enzyme E2 B; siRNA, small interfering RNA; MGMT, O6-methylguanine-DNA methyltransferase; pEGFP, plasmid expressing enhanced green fluorescence protein.

ubiquitination. These deactivated MGMT proteins may induce cell stress and lead to cell death (8). It was therefore hypothesized that higher UBE2B expression may be a prognostic factor for poor clinical prognosis in patients with NPC.

The present study confirmed that ubiquitination mediated by UBE2B is an important biological process in NPC. According to two public datasets, tumor tissues from patients with NPC had higher UBE2B mRNA expression levels than healthy nasopharyngeal tissues. Furthermore, *in vitro* assays suggested that UBE2B expression levels in NPC cells were higher than those in oral epithelial cells. The results of the clonogenic assay demonstrated that the cell growth ability was decreased in UBE2B-deficient NPC cells as compared with UBE2B-proficient cells. These findings support the role of UBE2B in NPC tumorigenesis. In the present NPC cohort of Chi-Mei Medical Center, no difference in important clinicopathological variables and UBE2B expression levels was observed between the high and low UBE2B expression groups. However, higher UBE2B expression was associated with poor DSS, DMeFS and LRFS. The results of the present survival analyses also support the role of higher UBE2B expression in regulating resistance to alkylating agents, such as cisplatin. Accordingly, high UBE2B expression may be employed as a risk factor for predicting poor prognosis of patients with NPC, particularly those undergoing cisplatin-based chemoradiotherapy. Cisplatin belongs to the group of alkylating chemotherapy drugs, which exert their anticancer activity by causing extensive DNA damage in tumor cells (29). Although several mechanisms of action may contribute to cisplatin resistance, DNA damage responses in tumor cells have a major role in the regulation of cisplatin-induced cytotoxicity. Accordingly, UBE2B may be involved in DNA damage responses in tumor cells, particularly those of cells exposed to alkylating chemotherapy drugs. In the mechanistic study performed in the present study, cisplatin cytotoxicity was increased in UBE2B-depleted NPC cells; however, MGMT expression attenuated cisplatin cytotoxicity induced by UBE2B knockdown. These results provide evidence that

UBE2B may regulate cisplatin sensitivity by targeting MGMT activity in NPC cells. In addition to the interaction between UBE2B and MGMT, a recent study demonstrated that treatment with UBE2B inhibitor is able to prolong the formation of γ-H2AX foci and recruitment of DNA repair proteins, such as 53BP1 and RAD51 (30). The present study also indicates that UBE2B downregulation may result in impairment of the DNA repair pathway. Taken together, high UBE2B expression levels would therefore lead to an offset in the effect of the alkylating agent, contribute to cisplatin resistance and predict poor clinical prognosis as a biomarker in patients with NPC treated with cisplatin-based chemoradiotherapy.

In the present study, clinical information and tumor tissues of 124 Taiwanese patients with NPC were compiled, and therefore, a relatively small sample size is the major limitation of the present study. In addition, selection bias may hinder the evaluation of the study results due to the retrospective nature of this study. However, the *in vitro* experiments indicated the role of UBE2B in the regulation of cisplatin cytotoxicity via MGMT activity in NPC cells. These findings may provide novel insight into therapeutic strategies for patients with NPC by targeting UBE2B expression.

In conclusion, the present study suggested that high UBE2B expression is a biological marker for poor prognosis in patients with NPC. The results of the present study support the role of UBE2B contributing to resistance mechanisms of alkylating agents in tumor cells, particularly NPC. Further research is an unmet requirement to overcome cisplatin resistance in patients with NPC and high UBE2B expression levels.

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**Availability of data and materials**

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

**Authors' contributions**

WCK, SHC and SYH designed and conceived the study. SHH and CLL performed the experiments. CLL, CYL, SWC, YXC and CHC contributed the resources. CYL, SWC, YXC and CHC collected and analyzed the clinical data. SWL and WTH interpreted the experimental data. CJT performed statistical analyses. CJT, SHC and SYH confirmed the authenticity of all the raw data. WCK and SYH wrote the manuscript. CJT, WTH and SHC revised the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The study was approved by the Ethics Committee of Chi Mei Medical Center in Taiwan (approval no. 10710-L01). Informed consent was obtained from all subjects involved in the study.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors indicate that they have no competing interests.

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