In Vitro Anticancer Activity of PI3K Alpha Selective Inhibitor BYL719 in Head and Neck Cancer

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Abstract. *Background/Aim: The purpose of the present study was to explore the antiproliferative effect of BYL719, a specific inhibitor for phosphatidylinositol 3-kinase (PI3K) p110α, in human head and neck cancer cell lines, as a single agent or in combination with the irreversible EGFR tyrosine kinase inhibitor, dacomitinib. Materials and Methods: Six head and neck cancer cell lines consisting of two PIK3CA mutant cell lines, SNU-1076 and Detroit562, and four PIK3CA wild-type cell lines, SNU-1066, SNU-1041, FaDu and SCC25, were analyzed. Results: The PIK3CA-mutant cell lines were more sensitive to BYL719 than the PIK3CA wild-type cell lines. Following BYL719 treatment, all PIK3CA wild-type cell lines, except for the SNU-1066 cell line, exhibited higher IC_{50} values compared to the PIK3CA mutant cell lines. Administration of BYL719 induced cell cycle G_0/G_1 arrest and resulted in increased apoptosis in a dose-dependant manner. Furthermore, the administration of BYL719 reduced the level of p-mTOR, p-AKT and p-S6 expression indicating the down-regulation of downstream signaling. Conclusion: BYL719, a PI3K alpha selective blocker, could be a promising factor in the treatment of head and neck cancer either as a single agent or in combination with dacomitinib.*

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer with an annual incidence of ~600,000 worldwide (1). Despite advances in the diagnosis and treatment of HNSCC, it still has of poor prognosis with a 5-year survival rate of 50% 1. In an attempt to improve the poor prognosis associated with HNSCC, recent studies of whole-exome sequencing in HNSCC have revealed a wide spectrum of unexpected genetic aberrations (2, 3).

A promising pathway, in terms of genetic aberrations, in HNSCC is the phosphatidylinositol 3-kinase (PI3K) signaling pathway. It is well-known that the PI3K signaling pathway regulates cell proliferation, cell survival and apoptosis (4, 5). Dysregulation or genetic aberration of the genetic components involved in the PI3K signaling pathway, including *AKT, PTEN* and *PIK3CA*, has been associated with cancer development and cancer progression (4, 5). The Class IA PI3K is a heterodimeric lipid kinase complex with two subunits, namely the p110α catalytic domain and the p85 regulatory domain. Upon ligand binding and receptor tyrosine kinase (RTK) auto-phosphorylation, PI3K is recruited to the cell membrane, binds to the intracellular arm of the RTK and catalyzes the conversion of phosphatidylinositol (4,5)-diphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) (4).

Recently, high frequencies of somatic mutations in the *PIK3CA* gene have been reported in HNSCC with frequent genetic aberration and amplification (6-8). More than 75% of these mutations are clustered in the helical (exon 9; E542K, E545K) and kinase domains (exon 20; H1047R) of the *PIK3CA* gene (6-8). These hot-spot mutations in the *PIK3CA* gene have been shown to elevate constitutive lipid kinase activity and lead to increased activation of the downstream AKT signaling pathway (9, 10). As PI3K is constitutively activated in *PIK3CA*-mutant tumors, PI3K appears to be an ideal target for drug development in the therapeutic treatment of *PIK3CA,* mutant tumors. A newly-developed PI3K inhibitor, BYL719 (11), has higher selectivity for the PI3K p110α subunit than any other PI3K subunits.

Dacomitinib (PF-00299804) is an orally administered, irreversible epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI). *In vitro* studies demonstrated that dacomitinib is a potent and specific inhibitor against EGFR,

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human epidermal growth factor receptor 2 (HER2) and HER4 tyrosine kinases (12). In the preclinical setting, dacomitinib was active not only against *EGFR* wild-type cancers but also in mutant models (13), including those harboring the *EGFRvIII* mutation, detected in up to 40% of HNSCC (14). Dacomitinib, as a single-agent, is also effective in recurred or metastatic HNSCC with a response rate of 12.7% (15).

The purpose of the present study was to investigate the *in vitro* antiproliferative effects of BYL719 in human head and neck cancer cell lines as either a single agent or in combination with an irreversible EGFR TKI, dacomitinib, and to determine the molecular mechanisms underlying the cell proliferation inhibition and the chemo-sensitizing effects. To gain a better understanding over the mechanism of growth inhibition, protein expression of downstream molecules, particularly in the EGFR signal transduction pathways, and the alteration of cell-cycle regulatory molecules were investigated.

Materials and Methods

Cell lines and culture. Six human head and neck cancer cell lines, consisting of two *PIK3CA*-mutant cell lines, SNU-1076 and Detroit562, and four *PIK3CA* wild-type cell lines, SNU-1066, SNU-1041, FaDu and SCC25, were purchased from the American Type Culture Collection (Manassas, VA, USA) and Korean Cell line Bank (Seoul, Korea). The SNU-1066, SNU-1041 and SNU-1076 cell lines were maintained in RPMI 1640 medium with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA). The Detroit562 and FaDu and SCC25 cell lines were maintained in American Type Culture Collection (ATCC) Eagle's modified essential medium (EMEM) with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) supplemented with 10% FBS (GIBCO). All cell lines were incubated under standard culture conditions (5% CO_2 at 37˚C).

Mutation analysis for head and neck cancer cell lines. The mutational profiles of head and neck cancer cell lines were screened *via* The Cancer Cell Line Encyclopedia (16) (CCLE) (http://www.broadinstitute.org/ccle). The CCLE is a compilation of gene expression, chromosomal copy number and massively parallel sequencing data from 947 human cancer cell lines. Mutation information was obtained by using both massively parallel sequencing of >1,600 genes and by mass spectrometric genotype screening (OncoMap). The *PIK3CA* mutation status of the HNSCC cell lines were obtained from CCLE. Among the 6 HNSCC cell lines, SNU-1076 and Detroit562 have a *PIK3CA* H1047R mutation in exon 20. The other 4 cell lines were *PIK3CA* wild types. The mutational profiles of the 6 HNSCC cell lines were re-confirmed by whole exome sequencing incorporated with another study. Detailed methods of the whole exome sequencing method are described in prior report (17).

Drugs and reagents. Both BYL719 and dacomitinib were purchased from Selleck Chemicals LLC (Houston, TX, USA). Both BYL719 and dacomitinib were initially dissolved in dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 10 mM and stored in small aliquots at –20˚C.

Cell growth-inhibition assay. A modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). The inhibitory concentration at 50% (IC_{50}) was calculated. Cells ($3\text{-}5\times10^3$) were seeded on 96-well plates and incubated for 24 h and then treated with drugs (BYL719 or dacomitinib) for 3 days at 37˚C. After drug treatment, CCK-8 solution was added to each well and the cells incubated for 4 hours at 37˚C. Absorbance was measured at 540 nm in an Eon™ Microplate Spectrophotometer (BioTek, Winoosk, VT, USA) in triplicate. Graphs were generated by nonlinear regression of the data points to a four-parameter logistic curve using the SigmaPlot software (Statistical Package for the Social Sciences, SPSS, Inc., Chicago, IL, USA). All data are expressed as mean±standard deviation (SD) obtained from at least three independent experiments.

Cell cycle analysis. Cells were plated in each culture dish and were treated with 0, 5, 10 mM/ml BYL719 for 72 h. Cell cycle analysis by flow cytometry was performed by detaching cells from culture plates with trypsin/ethylenediaminetetraacetic acid (EDTA), washing with phosphate buffered saline (PBS) and fixing in 75% ethanol. The pellet was re-suspended and incubated for 30 min in 5 μl PI (1 mg/ml) and RNase A 10 μl (10 mg/ml) in PBS. The suspension was then analyzed on a Becton Dickinson FACScan. The ratio of cells in the G0/G1, S and G2/M phase of cell cycle was determined by the relative DNA content per cell.

Annexin-V assay. Cells were treated with increasing doses of BYL719 (5 μM and 10 μM), incubated for 30 h, collected and concurrently stained with Annexin-V and 7-AAD (Becton Dickinson Biosciences, San Jose, CA, USA). Apoptotic cell death was determined by positive staining for Annexin-V and negative staining for 7-AAD using fluorescence-activated cell sorting analysis.

Western blot analysis and fluorescence in situ hybridization (FISH). Cells were re-suspended in lysis buffer (Cell Signaling Technology, Danvers, MA, USA), incubated on ice for 10 min and centrifuged for 15 min at 4˚C. Samples containing equal quantities of total protein were resolved on SDS–polyacrylamide denaturing gel, transferred to PVDF membranes and probed with antibodies, according to the manufacturer's procedure. Antibodies against EGFR, p-EGFR, HER2, p-HER2, PTEN, mTOR, p-mTOR, AKT, p-AKT, S6, p-S6, ERK, p-ERK and β-actin were purchased from Cell Signaling Technology. β-actin was used as the protein loading control. Detection was performed using an enhanced Lumi-Light Western Blotting Substrate kit (Roche, Indianapolis, IN, USA).

HER2 amplification of the 6 cell lines was determined by FISH using the PathVysion HER2 DNA Probe Kit (Vysis, Downers Grove, IL, USA). *HER2* FISH was performed by previous described methods (18). Cell lines were considered to be amplified if they had a copy number ratio of HER2/ chromosome 17 centromere (CEP17) >2.0

Determination of synergism and antagonism. To evaluate the effects of BYL719 administered in conjunction with dacomitinib, cells were treated with serial dilutions of each drug individually and with both drugs simultaneously at a fixed ratio of doses that corresponded to the individual IC_{50}. After 72 h of exposure, cell viability was measured using the MTT assay. The combination

index (CI) was calculated according to the Chou-Talalay method (19). Data were analyzed using the Calcusyn software (Biosoft, Ferguson, MO, USA). The CI index has been used for data analysis of two-drug combinations. CI<1, CI=1 and CI>1 indicate synergism, addictive effect and antagonism, respectively.

Results

Proliferation inhibition activity of BYL719 in head and neck cancer cell lines. Head and neck cancer cell lines (both PIK3CA-mutant and PIK3CA wild-type) were treated with increasing doses of BYL719 (no treatment, 0.1, 0.5, 1, 5, 10, 50 and 100 μM) for 72 h. The PIK3CA-mutant cell lines (SNU-1076 and Detroit562) were more sensitive to BYL719 with a significant decrease in cell proliferation in a BYL719 dose-dependent manner than the *PIK3CA* wild-type cell lines (SNU-1066, SNU-1041, FaDu and SCC25) (Figure 1). The IC_{50} values in the *PIK3CA*-mutant cell lines were 6.82 μM in SNU-1076 and 1.10 μM in Detroit562. The *PIK3CA* wild-type cell lines, except for the SNU-1066 cell line, exhibited higher IC_{50} values for BYL719 treatment (IC_{50} values: 1.13 μM, 20.65 μM, 19.67 μM and 49.30 μM in SNU-1066, SNU-1041, FaDu and SCC25, respectively).

Apoptotic effect and cell-cycle analysis of BYL719 treatment in head and neck cancer cell lines. The PIK3CA-mutant cell lines (SNU-1076, and Detroit562) and PIK3CA wild-type cell line SNU-1066 that showed exceptionally low IC_{50} values to BYL719 treatment were treated with BYL719 at various doses (no treatment, 5 and 10 μM) for 72 h. As measured by flow cytometry, BYL719 increased the sub-G_1 phase and induced G_1 arrest in the Detroit562 cells. Increased sub-G_1 phase and decreased S phase fraction arrest was also observed in the SNU-1076 and SNU-1066 cells (Figure 2A).

Apoptosis was detected by Annexin V staining in the Detroit562, SNU-1076 and SNU-1066 cells (Figure 2B). These cell lines showed increasing Annexin V staining cells in a BYL719 dose-dependent manner.

Effect of BYL719 on the PI3K downstream signaling. Changes in the protein expression of the downstream signaling pathway of PI3K were analyzed *via* western blot (Figure 3). The SNU-1076, Detroit562 and SNU-1066 cells were treated with various doses of BYL719 (0, 0.1, 1 and 10 μM) for 48 h. Protein expression of p-mTOR and p-AKT were reduced in a dose-dependent manner.

As SNU-1066, which is *PIK3CA* wild-type cell line, was sensitive to BYL719, we determined PTEN loss and *HER2* amplification. PTEN loss was not observed in SNU-1066 (Figure 3). However, HER2/CEP17 ratio of the SNU-1066 cell line was 4.40 in *HER2* FISH and *HER2* amplification was observed in SNU-1066.

Table I. *Combination index (CI) values of concurrent treatment with BYL719 and dacomitinib in head and neck cancer cell lines*.

	CI values	
	BYL719 (1 μM) + dacomitinib (0.01 μM)	BYL719 (10 μM) + dacomitinib (0.01 μM)
SNU-1076	0.223	0.308
Detroit562	0.140	0.266
SNU-1066	0.299	0.276
SNU-1041	0.107	0.353
SCC25	0.901	1.730
FaDu	0.118	0.315

CI<1 Synergistic; CI=1 additive; CI>1 antagonistic.

Synergistic effect of BYL719 combined with irreversible EGFR tyrosine kinase inhibitor, dacomitinib. The synergistic or additive effects of BYL719 combined with the irreversible EGFR inhibitor dacomitinib were evaluated after simultaneous exposure of dacomitinib to BYL719. As the IC_{50} value of dacomitinib was 4.73±0.21 μM, dacomitinib concentrations of a lower level IC_{50}, such as 0.01, 0.05 and 0.1 μM, were chosen for analysis of drug combination. When all cell lines were treated with combined dacomitinib and BYL719, the IC_{50} values of BYL719 decreased except for the SCC25 cell line, indicating that dacomitinib can produce a synergistic effect with BYL719 treatment (Figure 4). The synergistic activity of BYL719 combined with dacomitinib was seen not only in the *PIK3CA*-mutant cell lines but also in *PIK3CA* wild-type cell lines. SNU-1076, Detroit562, SNU-1066, SNUH-1041 and FaDu showed CI values <1 with the combination of BYL719 and dacomitinib, indicating a synergistic interaction (Table I).

Discussion

The present study shows the efficacy of BYL719, a novel PI3K-110α specific inhibitor, in head and neck cancer cell lines. In the present study, BYL719 inhibited cell proliferation by inducing apoptosis, particularly in *PIK3CA*-mutant head and neck cancer cell lines. In combination with dacomitinib, BYL719 treatment showed a synergistic cell proliferation inhibitory effect on *PIK3CA*-mutant cell lines, whereas this synergistic effect was not observed in the *PIK3CA* wild-type cell lines.

The recent elucidation of HNSCC genomics offers an opportunity to identify genotype-based treatment decisions. Recently, Stransky *et al*. (2) and Agrawal *et al*. (3) reported the mutational landscape of HNSCC using whole-exome sequencing. These two studies provided new insights into the genetic understanding of HNSCC and highlighted the high

PIK3CA mutation	mutant (H1047R)	mutant (H1047R)	wild type	wild type	wild type	wild type
Cell line	Detroit562	SNU-1076	SNU-1066	FaDu	SNU1041	SCC25
IC_{50} (μM)	1.10	6.82	1.13	19.66	20.65	49.30

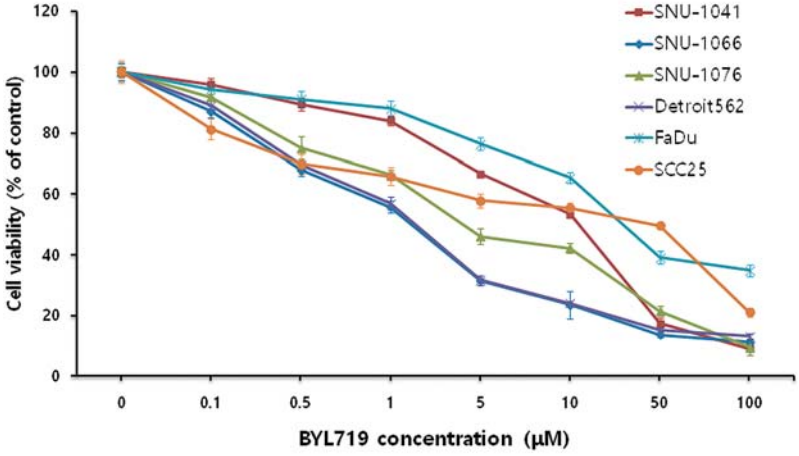

Figure 1. *Antiproliferative activity of BYL719 in various head and neck cancer cell lines. The six human head and neck cancer cell lines were treated with increasing concentrations of BYL719 (0, 0.1, 0.5, 1, 5, 10, 50 and 100 μM) for 72 h. The IC_{50} values and cell viability using an MTT assay were determined by measuring the absorbance at 540 nm in a microplate reader. Each value represents the means of 12 replication experiments.*

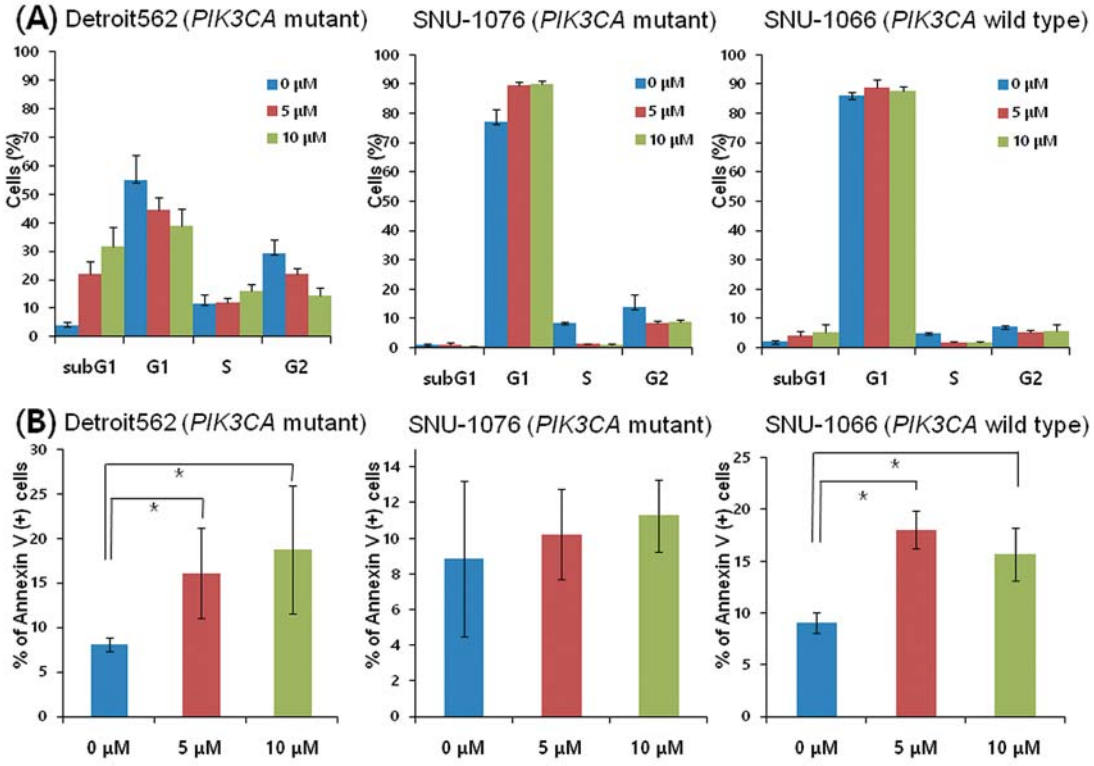

Figure 2. *Effects of BYL719 on cell cycle. (A) PIK3CA mutant cell lines (SNU-1076 and Detroit562) and the PIK3CA wild type cell line SNU-1066 were treated with BYL719 (0, 5 and 10 μM) for 72 h. Proportions of cells in the G_1, S and G_2-M phase were quantified; total percentages of G_1, S and G_2-M phases are presented as 100%. Treatment with BYL719 caused accumulation of Detroit562 cells in the sub-G_1 phase. (B) For assessing the apoptosis of SNU-1076 and Detroit562 cells, the staining of Annexin V-phycoerythrin by fluorescence-activated cell sorting analysis Caliber was performed. Both the SNU-1076 and Detroit562 cells showed increasing annexin V staining cells in an increasing concentration of BYL719. The data are representative of six independent experiments. *$p<0.001$.*

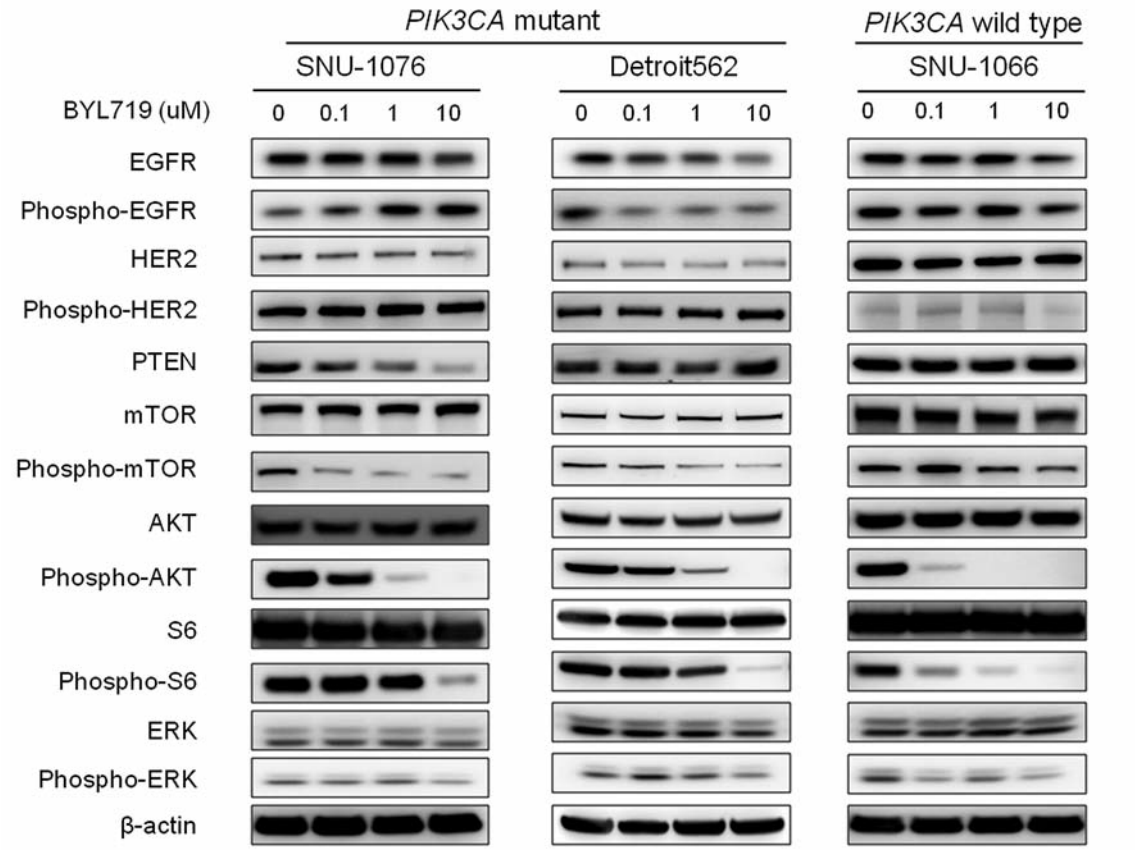

Figure 3. *Western blotting was conducted for PI3K downstream signaling after treatment with BYL719. Detroit562, SNU-1076 and SNU-1066 cells were treated with BYL719 (0, 0.1, 1 and 10 μM) for 48 h. Downstream signaling molecules (mTOR, AKT, S6) and the phosphorylated forms of each protein were observed with the same treatments. The protein expression of p-mTOR and p-AKT were down-regulated.*

frequency of mutations in tumor suppressors genes including: *TP53; CDKN2A; NOTCH1; NOTCH2; NOTCH3* and *FAT1* (2, 3). Among the various altered genes observed in whole-exome sequencing, the most targetable oncogene with sufficient mutation frequency was *PIK3CA*. The *PIK3CA* mutations comprised from 8.0% to 30.5% of HNSCC (2, 3, 6-8, 20). In addition, *PIK3CA* mutations occur at a high frequency in HPV-positive oropharyngeal cancer (7), of which the incidence is rising rapidly (21). Furthermore, more than 75% of *PIK3CA* mutations are hot-spot mutations in the helical (exon 9; E542K, E545K) and kinase domains (exon 20; H1047R) of the gene; these hot spot mutations constitutively activate the PI3K pathway. Activation of the PI3K pathway offers the possibility for personalized therapy with *PIK3CA* pathway inhibitors to improve the treatment outcomes of HNSCC.

Somatic mutations in *PIK3CA* have been identified in a variety of human cancers, including breast, colon, endometrial cancers and glioblastomas (22). Most of these mutations cluster to two hot-spot regions in exon 9, which encodes the helical domain of p110α, and in exon 20, which encodes the catalytic domain of p110α. These mutations de-repress an inhibitory interaction between the N-terminal SH2 domain of p85 and the p110α catalytic subunit (23). These *PIK3CA* mutants lead to increased oncogenic potential *in vitro* and *in vivo* (24, 25) by causing constitutive activation of the PI3K pathway in the absence of growth factors.

The findings of the current study show that BYL719 possesses a high cytotoxicity in PIK3CA-mutant head and neck cancer cell lines. The growth inhibition of the three head and neck cell lines occurred in the micromolar range (1.10-6.82 μM range) of BYL719 and no cell line examined in the present study was resistant to a single treatment with this agent. This is comparable with previous reports of BYL719 treatment in breast cancer cell lines (26) and myeloma cell lines (27). In addition, the antitumor effects of BYL719 treatment in xenograft models have previously been reported (26). The data of the present study also suggest that BYL719 can have synergism with the irreversible EGFR TKI, dacomitinib, further inhibiting carcinoma cell

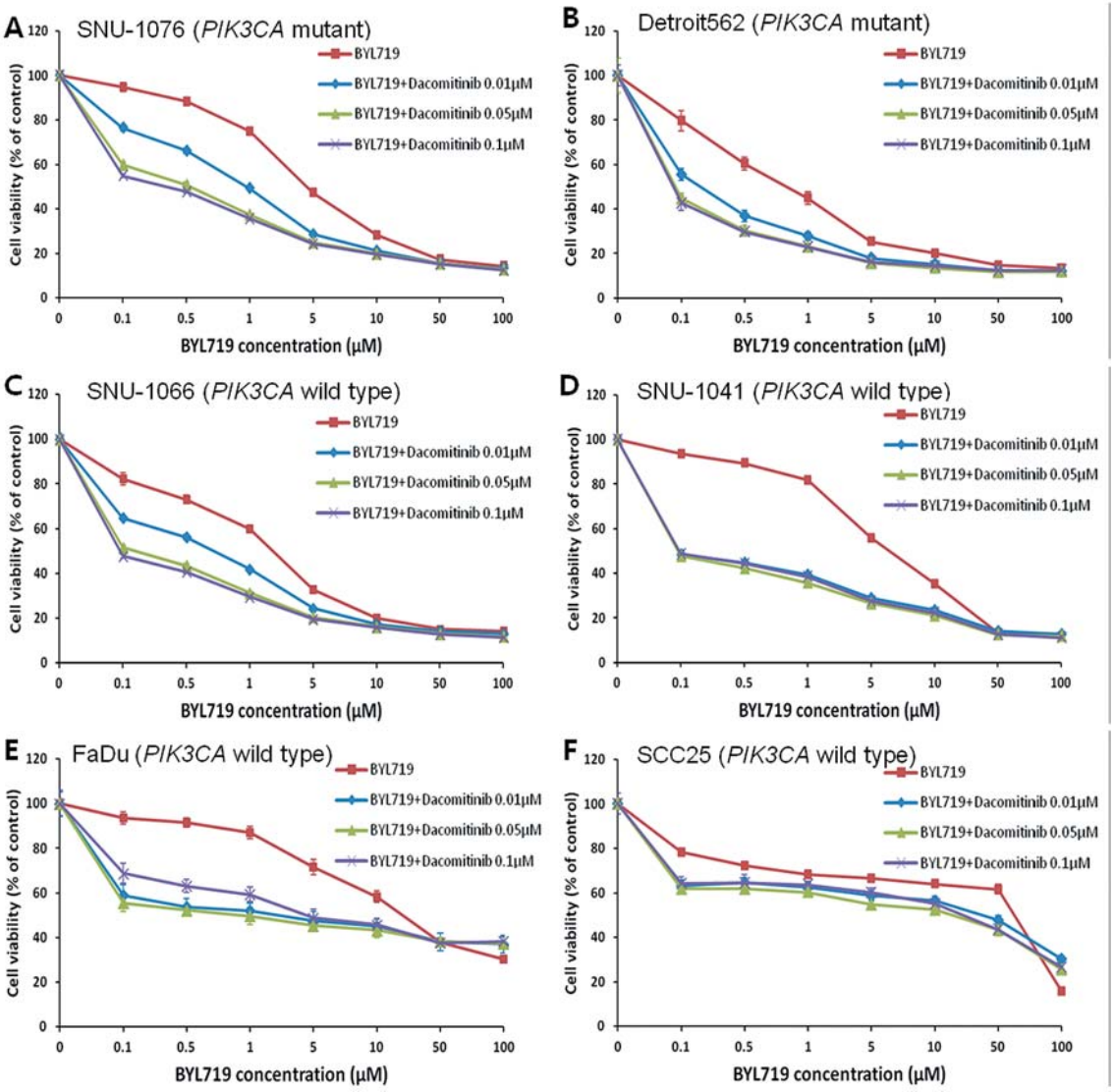

Figure 4. *Analysis of synergistic interactions between BYL719 and dacomitinib. IC_{50} values decreased with combined dacomitinib and BYL719 treatment. The IC_{50} value of BYL719 was markedly decreased, particularly in the two PIK3CA mutant cell lines (Detroit562, SNU-1072) in a dose-dependent manner. The IC_{50} value of BYL719 was not changed in the SCC25, PIK3CA wild-type, cell line.*

proliferation. As HNSCC expresses high levels of EGFR on the cell surface (28) and dacomitinib, as a single agent, is also effective in preventing recurred or metastatic HNSCC (15), BYL719 in combination with dacomitinib may be a promising therapeutic approach for *PIK3CA*-mutant HNSCC.

In the present study, the *PIK3CA*-mutant cell lines showed better sensitivity to BYL719 treatment compared to the PIK3CA wild-type cell lines. Interestingly, one *PIK3CA* wild-type cell line, SNU-1066, also had good sensitivity to BYL719 treatment as indicated by induced apoptosis even when compared with the two *PIK3CA*-mutant cell lines. It is of note that BYL719 can be effective in *PIK3CA* wild-type cell line. Synergistic interaction between BYL719 and dacomitib were observed in both *PIK3CA*-mutant and wild-type cell lines.

Such findings of the current study suggest that, besides the *PIK3CA* mutation, there may be additional mechanisms involved in HNSCC, such as upstream pathway activation or loss of PTEN or mutation of *PTEN*. In our study, it was suggested that HER2 amplification and the activation of upstream pathway of PI3K were the reason for BYL719 sensitivity in the *PIK3CA* wild-type cell line. A predictive factor for BYL719 in *PIK3CA* wild-type cell line needs to be elucidated in order to establish the optimal cell type candidate for BYL719 treatment.

In conclusion, BYL719 has cell proliferation inhibitory effects in several head and neck cancer cell lines, in particular in *PIK3CA*-mutated head and neck cancer cell lines. The results of the present study provide evidence that BYL719 is a potential target in the treatment of HNSCC with *PIK3CA* mutation. Future clinical trials of BYL719 alone or in combination with an irreversible EGFR TKI, such as dacomitinib, in HNSCC, are warranted.

Conflicts of Interest

The Authors have no conflict of interest.

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