

From the Klinik für Mund-, Kiefer- und Gesichtschirurgie  
(Director: Prof. Dr. med. Dr. Med. Dent. J. Wiltfang)  
at the University Medical Center Schleswig-Holstein, Campus kiel,  
at Kiel University

EFFECT OF CILENGITIDE AND VISMODEGIB  
ON PRIMARY CELL CULTURES AND CELL  
LINES OF HEAD AND NECK SQUAMOUS  
CELL CARCINOMA (HNSCC) AFTER  
TARGETING INTEGRIN AND HEDGEHOG  
PATHWAYS

Dissertation  
to acquire the doctoral degree in dentistry (Dr. med. dent)  
at the Faculty of Medicine  
at Kiel University

presented by  
LIEFEN ZHANG  
from NinBo, Zhejiang, P.R China  
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## Abbreviations

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HNSCC	Head and neck squamous cell carcinoma
ATCC	American type culture collection
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DXM	Dexamethasone
EB	Ethidium Bromide
ECM	Extracellular Matrix
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HCl	Hydrogen Chloride
M	Mole
mM	Millimole
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium
nM	Nanomole
$\mu$ M	micromole
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P/S	Penicillin/ treptomycin
PH	Hydrogen Ion Concentration
SKY	Spectral karyotyping hybridization
STR	Short tandem repeat
SHH	Sonic Hedgehog
TNM	TNM Classification of Malignant Tumors
U	Unit

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# 1. Introduction

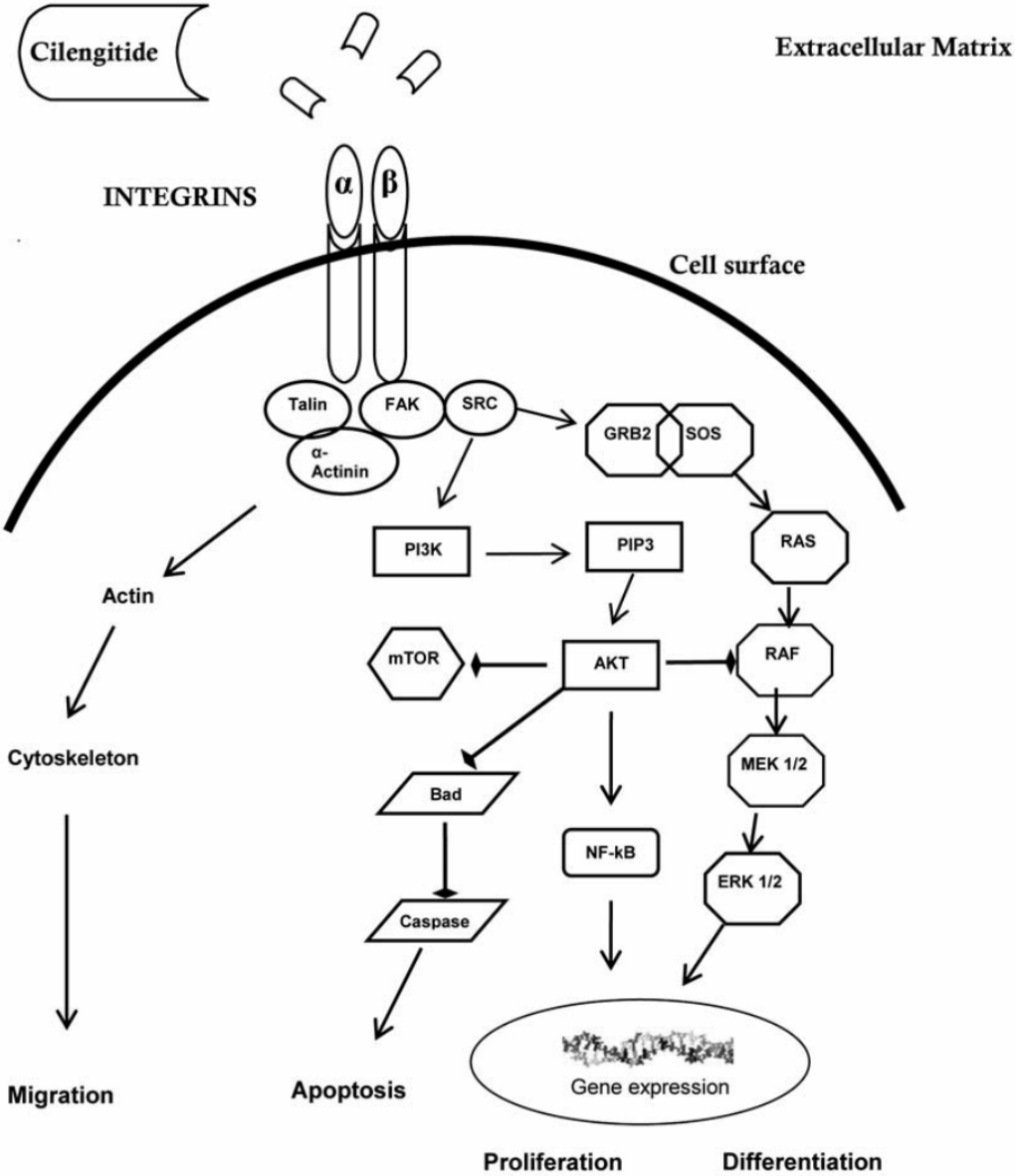
Head and neck squamous cell carcinoma (HNSCC) is one of the six malignant lesions worldwide with the highest incidence rate, and accounts for 90% of all oral cancers (Jemal et al., 2011). Current curative treatment approaches include surgery, irradiation and irradiation in combination with chemo- or immunotherapy, depending on the stage of the lesion. Due to the low 5-year survival rate and the high recurrence rate of HNSCC, new chemotherapeutic strategies are of high clinical interest. The discovery and development of effective chemotherapeutic agents for HNSCC is essential. Despite tremendous research efforts, the prognosis for affected patients remains dismal, particularly for those with locally advanced disease. Additionally, the impact on their quality of life is substantial (de Bree et al., 2000). Several factors may contribute to these disappointing results, including acquired drug resistance and tumor hypoxia (Denaro et al., 2012).

## 1.1 Integrin pathway

Integrins are heterodimeric transmembrane glycoproteins composed of two subunits  $\alpha$  and  $\beta$ , together forming more than 24 different proteins using 18  $\alpha$  and 8  $\beta$  subunits. They have a crucial role in cell-cell adhesion and cell interaction with the extracellular matrix (ECM). Integrins bind with specific ECM proteins, such as fibronectin, laminin, collagen, vitronectin and fibrinogen, through specific ligands that recognize the Arg-Gly-Asp (RGD) tripeptide motif (Seguin et al., 2015). The  $\alpha$  subunit, consisting of about 1100 amino acids, is the largest subunit and is composed of chains of different length held together by two disulfide bridges. The  $\beta$  subunit consists of about 800 amino acids, with the exception of the  $\beta$ 4 subunit, which contains about 1750 amino acids. Both the  $\alpha$  and  $\beta$  subunits have a small transmembrane region, a large extracellular domain and a small cytoplasmic tail. The cytoplasmic domain is crucial for the regulation of integrin activity. It controls the integrin affinity state and its ECM ligand-binding activity, and promotes cellular responses upon extracellular ligand binding. After activation of the cytoplasmic domain by its extracellular ligand, integrins, which lack intrinsic kinase activity, cluster to form cell membrane focal adhesion complexes. Focal adhesion kinase (FAK) is recruited and autophosphorylated at these sites, in turn activating signaling pathways through phosphatidylinositol 3-kinase (PI3K), nuclear factor kappa B

(NF- $\kappa$ B), sarcoma tyrosine kinase (SRC) and extracellular signal-regulated kinase (ERK/MAPK) (Fig. 1) (Reardon and Cheresh, 2011).

**Figure 1:** Schematic representation of main signaling pathways activated by integrins(Reardon and Cheresh, 2011).



This leads to the regulation of several cellular processes, including cell motility and invasion, remodeling of the ECM by means of localization proteases, cell growth through adhesion-dependent control of proliferation, and cell signaling by cross-talk with growth factors and cytokine receptors. In addition, integrins are involved in several steps of normal immune-cell function, including T-cell activation and lymphocyte adhesion to endothelial cells (Reardon



and Cheresch, 2011), and modulate apoptosis, regulating the activity of pro-apoptotic proteins, such as caspase, through a mechanism called integrin-mediated death (Buckley et al., 1999). However, the exact molecular mechanisms and pathways involved in their actions remain unclear.

Cilengitide is a cyclized Arg-Gly-Glu (RGD)-containing pentapeptide that selectively blocks activation of the  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins (Goodman et al., 2002). Cilengitide has been used for inhibiting rapid growth of highly vascularized tumors, highlighting the potential of this new therapeutic agent (Raguse et al., 2004). It may have a direct effect on the migration, attachment and viability of tumor cells. Studies of cilengitide have focused mainly on glioblastoma (MacDonald et al., 2008). No data have been published on the effect of cilengitide on primary HNSCC cell cultures.

In previous studies, most cell lines were purchased from commercial companies (Liu et al., 2016, Sasahira et al., 2016). These cell lines may have already differentiated and lost some of their potential characteristics as tumor cells. In contrast, primary cell cultures may retain their internal biological characteristics (Manikandan et al., 2016). Accordingly, we isolated and identified primary HNSCC cell cultures, and evaluated the effects of cilengitide on those cultures and on cell lines. The application of drugs to the cell lines or primary cell cultures may produce different results.

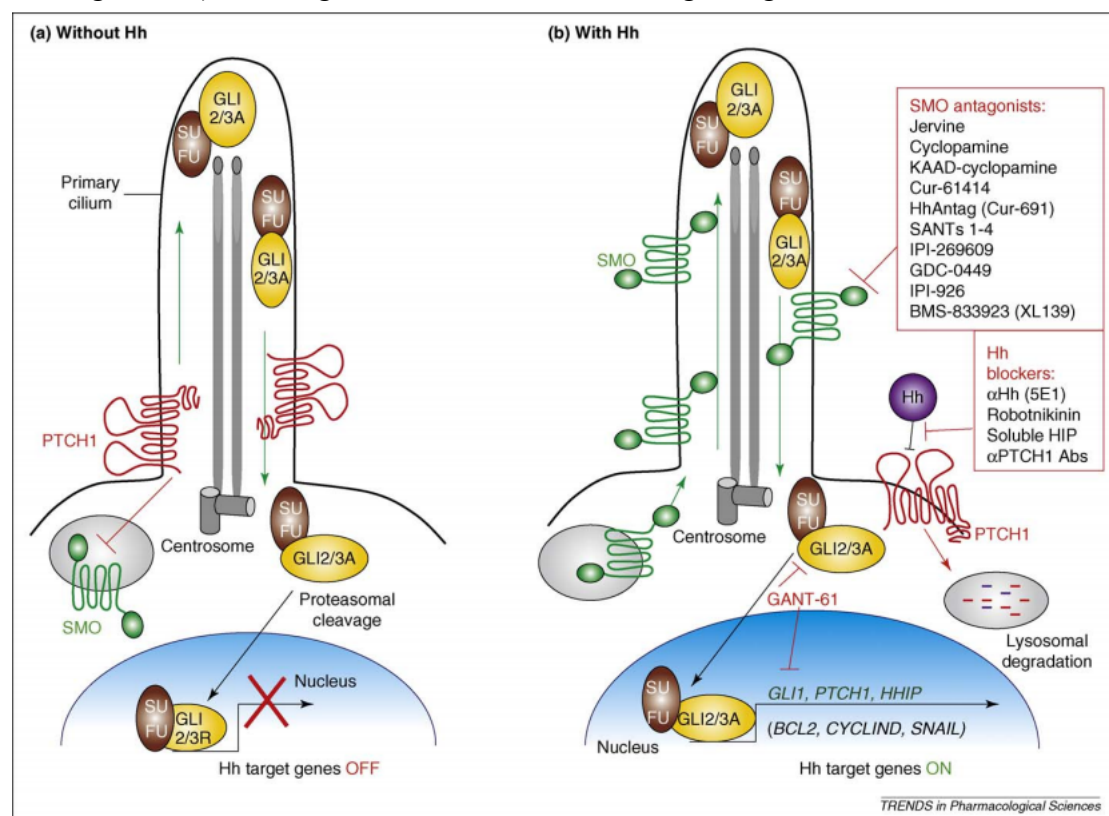
## **1.2 Hedgehog signal pathway**

Recently, growing evidence has suggested that aberrant activation of the Hedgehog signaling (HH) pathway in squamous cell carcinoma (SCC) plays a critical role during cancer development (Yan et al., 2011, Liebig et al., 2017). Canonical HH signaling is initiated by the binding of HH ligands (Sonic, Indian and Desert hedgehog) to a 12-transmembrane receptor Patched (PTCH), which relieves the catalytic inhibition of Smoothed (SMO), a 7-transmembrane G-protein coupled receptor (GPCR)-like signal transducer. SMO de-repression triggers a series of intracellular events, resulting in the activation of downstream target genes through the zinc finger transcription factors GLI1, GLI2 and GLI3 (Lum and Beachy, 2004). GLI activation is complex with regulation at both the transcriptional and post-translational levels (Ruiz i Altaba et al., 2007). GLI2 appears to be the primary activator of HH signaling in cancer, with GLI1 as a transcriptional target of GLI2 (Bai et al., 2002, Ikram et al., 2004). GLI2 and GLI1 also induce transcription of overlapping and distinct sets of downstream targets (Eichberger et al., 2006). Several components of the HH pathway (PTCH,

GLI1, GLI2, HHIP) are GLI transcriptional targets that induce positive or negative feedback (Bigelow et al., 2004). GLI targets mediate various cellular responses, notably enhanced cell proliferation and survival, by upregulating D-type cyclins and anti-apoptotic proteins (Bigelow et al., 2004). Finally, phosphatidylinositol 3-kinase (PI3K)/AKT and RAS-MEK signals have been described as non-canonical HH activators in cancer (Nolan-Stevaux et al., 2009, Riobo et al., 2006). Upon HH binding, PTCH1 translocates out of the primary cilium, losing its ability to inhibit SMO, which moves into the cilium, thus stimulating the pathway and preventing GLI2 and GLI3 cleavage. Activated GLI2 and to a lesser extent GLI3 then bind to GLI-promoters in the nucleus and stimulate transcription of the ubiquitous mammalian target genes, GLI1, PTCH1, and HHIP, and also cell-type specific genes, which can include BCL2, Cyclins and Snail. PTCH1 and HH are internalized and degraded in lysosomes (Fig. 2).

**Fig. 2:** The basic components of the mammalian Hedgehog signaling pathway. (a) In the absence of HH ligands. (b) With HH ligands. Shown in red are various Hedgehog signaling pathway antagonists (Scales and de Sauvage, 2009).

Dysregulation of the HH pathway has been implicated in a variety of cancers (Scales and de Sauvage, 2009). Although the involvement of HH signaling in SCC is well established (Yan



et al., 2011, Liebig et al., 2017), the role of this pathway remains poorly understood.

However, multiple lines of evidence point to the potential importance of HH signaling in SCC. Immunohistochemical studies of patient specimens reported the overexpression of HH signaling components (Cavicchioli Buim et al., 2011, Dimitrova et al., 2013, Srinath et al., 2016, Kuroda et al., 2017). HH signaling has also been implicated in squamous cancer of other organs (Szkandera et al., 2013, Huang et al., 2014). Despite these studies, very little is known regarding the specific role of HH signaling in regulating cellular survival and proliferation in HNSCC. Targeted inhibitors of the HH pathway have become available recently. Because of its accessibility on the membrane and its importance in regulation of the pathway, SMO has been the primary focus in the development of small molecule inhibitors of the HH pathway. Vismodegib (GDC-0449) is an orally administered agent that selectively suppresses SMO activity, and was the first SMO inhibitor to progress to clinical trials. It has produced promising antitumor responses in patients with advanced basal cell carcinoma and medulloblastoma (Rudin et al., 2009, Von Hoff et al., 2009), but resistance has been reported (Dijkgraaf et al., 2011, Yauch et al., 2009). The resistance to SMO inhibitors highlights the therapeutic need to target downstream effectors to maintain robust on-target responses. To our knowledge, there is still a lack of data from clinical trials regarding the effect of vismodegib on HNSCC.

### **1.3 The hypothesis of this study**

Due to the low 5-year survival rate and the high recurrence rate of HNSCC, new chemotherapeutic strategies are of high clinical interest. Integrins are important adhesion molecules and play a regulatory role in many critical biological processes such as cell proliferation, invasion, apoptosis, metastasis, and angiogenesis in squamous cell carcinoma. Cilengitide is a cyclized RGD containing pentapeptide that selectively blocks activation of the integrins. Studies of cilengitide have focused mainly on glioblastoma (MacDonald et al., 2008). No data have been published on the effect of cilengitide on primary HNSCC cell cultures.

Recently, growing evidence has suggested aberrant activation of the hedgehog signal pathway in squamous cell carcinoma plays a critical role during cancer development. Vismodegib (GDC-0449) is an orally administered agent that selectively suppresses SMO activity of the HH pathway. HH signal pathway has also been implicated in squamous cancer of other organs (Szkandera et al., 2013, Huang et al., 2014). Despite of these studies, very little is known

regarding the specific role of HH signaling in regulating cellular survival and proliferation in HNSCC.

Most previous studies used cell lines purchased from commercial companies. These cell lines may already have differentiated and lost some of their potential characteristics as tumor cells.

Therefore, the aim of the whole research study was to

1. Isolate and identify primary HNSCC cell cultures
2. Evaluate the effects of cilengitide and vismodegib on the proliferation of cells
3. Demonstrate the change of gene expression after application of drugs to the cell lines or primary cell cultures respectively.

## 2. Material and methods

### 2.1 Materials

Material	Catalog number	Manufacturer
Tissue culture plate 96-well	83.1835.300	SARSTEDT, Numbrecht
Tissue culture plate 6-well	83.1839.300	SARSTEDT, Numbrecht
Falcon Tube 15 ml	62.553.042	SARSTEDT, Numbrecht
Falcon Tube 50 ml	62.547.254	SARSTEDT, Numbrecht
Flask T75	83.3911.002	SARSTEDT, Numbrecht
Serological Pipette 25 ml	86.1685.001	SARSTEDT, Numbrecht
Serological Pipette 10 ml	86.1254.001	SARSTEDT, Numbrecht
Serological Pipette 1 ml	86.1251.001	SARSTEDT, Numbrecht
Biosphere Filter Tips 0.5-10 $\mu$ l	70.115.210	SARSTEDT, Numbrecht
Biosphere Filter Tips 2-100 $\mu$ l	70.760.212	SARSTEDT, Numbrecht
Biosphere Filter Tips 100-1000 $\mu$ l	70.762.211	SARSTEDT, Numbrecht
Combitips advanced 1 ml	0030089545	EppendorfBiopur, Hamburg
Combitips advanced 5 ml	0030089669	EppendorfBiopur, Hamburg
CryoRure Tube 1.8 ml Nunc Lab-Tek II	72.379	SARSTEDT, Numbrecht
Chamber Slide	154534	Thermo Scientific, Rochester, USA

## 2.2 Reagents

Reagent	Catalog number	Manufacturer
Primer SHH	QT01156799	QIAGEN, Hilden, Germany
Primer GLI1	QT00060501	QIAGEN, Hilden, Germany
Primer SUFU	QT00050260	QIAGEN, Hilden, Germany
Primer SMO	QT00050701	QIAGEN, Hilden, Germany
Primer PTCH	QT00075824	QIAGEN, Hilden, Germany
Primer ITGAV	QT00051891	QIAGEN, Hilden, Germany
Primer ITGB3	QT00044590	QIAGEN, Hilden, Germany
Primer ITGB5	QT00044520	QIAGEN, Hilden, Germany
Primer GAPDH	QT00079247	QIAGEN, Hilden, Germany
Cell Proliferation kit I (MTT)	11645007001	Roche Applied Science, Indianapolis, USA
Cell Proliferation ELISA kit (BrdU)	11647229001	Roche Applied Science, Indianapolis, USA
DNA Ladder 25-700 bp	SM1191	Thermo Scientific, NY, USA
Dulbecco's MEM (DMEM)	T041-01	Biochrom AG, Berlin, Germany
Ethidium Bromide	1.11608.0010	Merck K GaA, Germany
FCS	S0115	Biochrom AG, Berlin, Germany
Anti-CD90 / Thy1 antibody	ab133350	ABcam, San Francisco, CA, USA
Anti- Cytokeratin antibody	ab7753	ABcam, San Francisco, CA, USA
Anti-SHH antibody	ab53281	ABcam, San Francisco, CA, USA
Anti-Vimentin antibody	ab20346	ABcam, San Francisco, CA, USA

PBS powder without Ca <sup>2+</sup> , Mg <sup>2+</sup>	L182-10	Biochrom AG, Berlin, Germany
Biotin Blocking System	X0590	Dako, Copenhagen, Denmark
LSAB+ System-HRP	K0679	Dako, Copenhagen, Denmark
Molecular Biology Agarose	161-3102	Bio-Rad Laboratories, CA, USA
Penicillin/ Streptomycin	A2213	Biochrom AG, Berlin, Germany
Propidium Iodide	P4710	Sigma-Aldrich Chemistry GmbH, Steinheim, Germany
QuantiTect SYBR Green RT- PCR kit	204243	Qiagen, Hilden, Germany
Rneasy plus Mini kit	74134	Qiagen, Hilden, Germany
QuantiTect reverse transcription kit	205311	Qiagen, Hilden, Germany
Cilengitide	S707702	Selleck chemicals, Houston, USA
Vismodegib	S603832	Selleck chemicals, Houston, USA
KaryoMAX® Colcemid™ Solution	15210-040	GIBCO, CA, USA

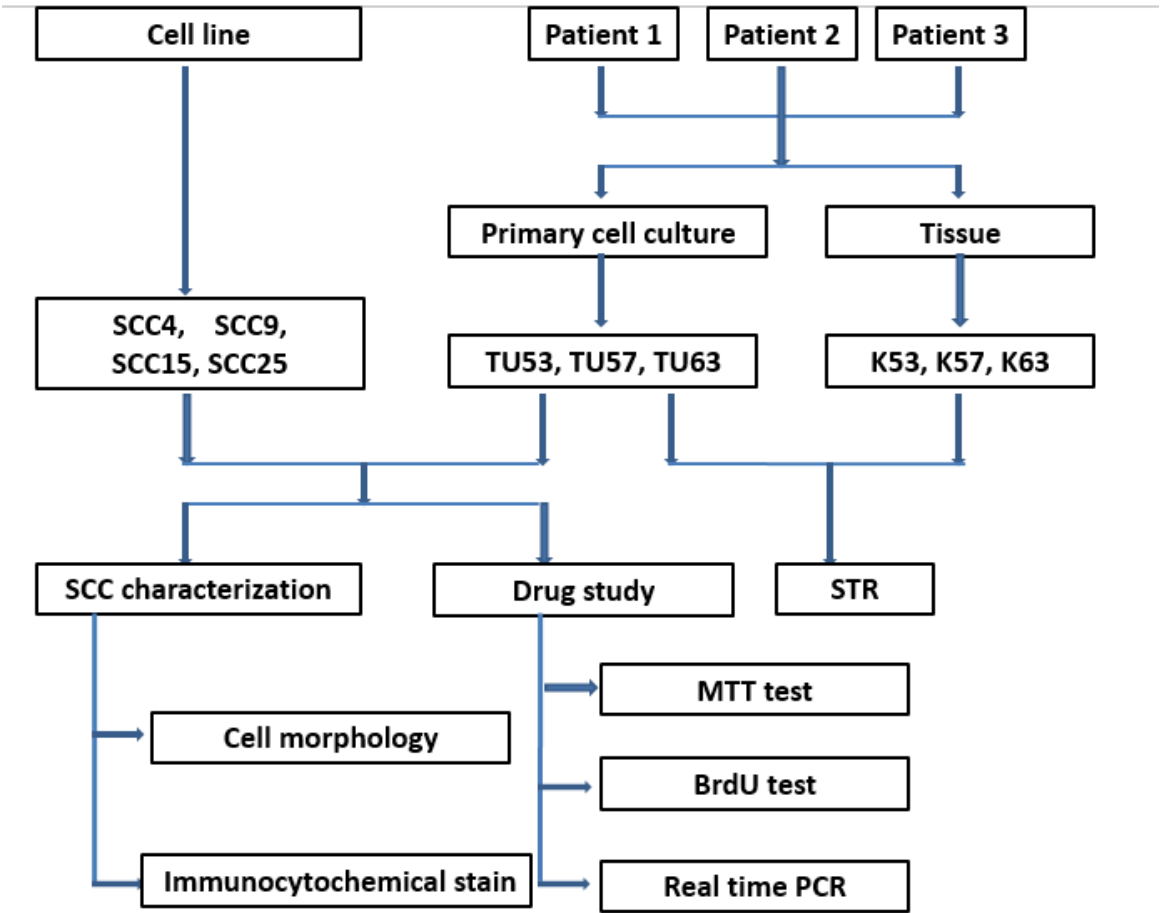
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## 2.3 Instruments

Instrument	Manufacturer
8-channel Pette with adjustable volume	Eppendorf AG, Hamburg, Germany
BioDocAnalyze System	Biometra, Goettingen, Germany
BLAUBRAND-Hemocytometer	Brand GmbH CO. KG, Wertheim, Germany
Centrifuge 5702R	Eppendorf AG, Hamburg, Germany
Coolpix MDC lens	Nikon Corporation, Tokyo, Japan
Einfrierbox (cry®box)	Nunc A/S, Roskilde, Denmark
Eppendorf single-channel pipette 1 - 10 µl	Eppendorf AG, Hamburg, Germany
Eppendorf single-channel pipette 10 - 100 µl	Eppendorf AG, Hamburg, Germany
Eppendorf single-channel pipette 100 – 1000 µl	Eppendorf AG, Hamburg, Germany
Emitech K-850 Critical Point Drier	Emitech Products Inc, Butte Creek, Texas, USA
Filtration device SM 16510	Sartorius GmbH, Germany
GX-600	A&D Company, Ltd, Japan
Heraeus HERA-cell 240 CO <sub>2</sub> -Incubator	Kendro Laboratory Products GmbH, Hanau, Germany
Invert-Microscope	Leitz GmbH, Wetzlar, Germany
Light Cycler® 2.0	Roche, Mannheim, Germany
Microscope M 420	Wild Heerbrugg, Switzerland
Microphoto-FXA	Nikon Corporation, Tokyo, Japan
Mini Gel Migration Trough	Cosmo Bio Co. Ltd, Germany
Nikon Coolpix 5000	Nikon Corporation, Tokyo, Japan
SOFTmax Pro	Molecular Devices, Sunnyvale, CA, USA
SPECTRA MAX Plus	Molecular Devices, Sunnyvale, CA, USA
Vortexer: Vortex Genie 2	Bender&Hobein, Schweiz, Germany



## 2.4 experimental scheme



**Fig. 3:** Experimental scheme of the whole research study.

## **2.5 Isolation and culture of SCCs**

### **2.5.1 Cell lines**

Four tongue squamous cell carcinoma cell lines (SCC4, SCC9, SCC15 and SCC25) were obtained from the American Type Culture Collection. The cells were cultured in DMEM medium containing 10% fetal bovine serum in an incubator with 5% CO<sub>2</sub> at 37 °C. The medium was refreshed every three days. The cells were passaged when they reached a confluence of 80-90%. The culture medium was removed first. The cell layer was briefly rinsed with phosphate buffered saline (PBS) to remove all traces of the serum, which contained trypsin inhibitor. 3 ml of 0.25% (w/v) trypsin solution were added to the flask and cells were observed under an inverted microscope until the cell layer was dispersed (usually within 5 to 15 min). The suspension was transferred to a sterile 50-ml tube and centrifuged at 1200 rpm for 5 min. After the removal of the supernatant, the cells were resuspended and dispensed into new 75-cm<sup>2</sup> flasks and incubated at 37 °C.

### **2.5.2 Primary cell cultures**

Tissues K53, K57 and K63 were obtained, respectively, from three patients with HNSCC who underwent surgery in the Department of Oral and Maxillofacial Surgery, Kiel University between 2011 and 2013. All patients were treated with surgery with curative intent and had negative resection margins. The study was approved by the Institutional Review Board. After surgical resection, tissues were cut into several pieces. Remaining tissue was put into a liquid nitrogen container for long-term storage. Three small pieces were each promptly transferred to a sterile dish with penicillin/streptomycin and hydrocortisone-containing medium, cut into small pieces and transferred to a T75 flask with 10 ml of medium. The tissue was incubated in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. The medium was changed after 7 days and then every 3 days. Tissue fragment and non-adherent blood cells were discarded. When cells reached 80% confluence, through time-controlled passage, the primary cell cultures TU53, TU57 and TU63 were purified.

**Table 1:** Information about the primary cell cultures

Patient ID	Primary cell culture	Gender	Age (years)	Histological grading	TNM stage	Tumor Site
1	TU53	female	54	Moderately differentiated	T2N0M0	tongue
2	TU57	female	42	Moderately differentiated	T2N2M0	tongue
3	TU63	male	52	Poorly differentiated	T3N0M0	buccal

### 2.5.3 Freezing and long-term storage of cells

SCCs were harvested at 80% confluence for freezing. To collect cells, 5 ml of 0.25% trypsin were added to detach the cells. After centrifugation, the pellet was resuspended in warm cryopreservation medium containing 90% fetal bovine serum and 10% dimethyl sulfoxide (DMSO) in a final amount of 1 ml/vial. The vials were maintained in an alcohol freezing container and transferred to a -80 °C refrigerator in which the vials were cooled slowly, by about 1 °C every minute, until they reached -80 °C. The cells were stored at -80 °C overnight before being transferred to a liquid nitrogen container for long-term storage. Vials of cells were stored in the vapor phase of the liquid nitrogen tank. For cell resuscitation, the cells from a frozen vial were rapidly thawed to 37 °C, and immediately centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the pellet suspended in a T75 flask with 10 ml DMEM medium containing 10% fetal bovine serum.

## **2.6 HNSCC characterization**

### **2.6.1 Immunocytochemical staining**

After passage, HNSCCs were counted using a hemocytometer, adjusted to a concentration of  $5 \times 10^3/\text{ml}$  and seeded in 6-well chamber slides with 0.5 ml of medium in each well. The cells were cultured for 4-5 days until they reached 80-90% confluence. Then medium was discarded and the cells were washed twice with warm PBS. The chambers were removed from the slides using separator keys. The slides were dried in air at room temperature (RT) for 1 h, placed in acetone for 10 min, dried in air for 0.5 h, and then stored at  $-20^\circ\text{C}$ .

#### **2.6.1.1 CD90**

Slides were put in methanol for 10 min and then distilled water. Endogenous peroxidase activity was blocked with endogenous peroxidase blocking buffer (20  $\mu\text{l}$  per well) for 10 min at RT. The slides were then washed in PBS. Endogenous avidin/biotin activity was blocked with endogenous avidin/biotin blocking buffer according to the instructions accompanying the kit. Each well was incubated at  $4^\circ\text{C}$  overnight with 20  $\mu\text{l}$  of CD90 antibody diluted 1:100 in 1% bovine serum albumin (BSA) in PBS. Slides were washed three times with PBS for 5 min each during incubation. Each well was incubated with 20  $\mu\text{l}$  biotinylated secondary antibody for 30 min at RT. Slides were washed three times in PBS with 5 min each during incubation. 20  $\mu\text{l}$  streptavidin-HRP were applied to each well on slides and incubated for 30 min at RT. Slides were washed three times for 5 min in PBS. Working DAB substrate solution was mixed and incubated on the slides for a maximum of 5 min at RT. The development of the color reaction was stopped by washing with PBS. Slides were counterstained with hematoxylin for 1 min and rinsed in flowing water for 10 min. The slides were mounted with mounting medium. Sections without primary antibodies served as negative controls. The sections were examined and scored using a microscope at 400X magnification.

#### **2.6.1.2 Cytokeratin**

The procedure was the same as that described for CD90 except for the incubation conditions of the primary and secondary antibodies. Each well was incubated at RT for 1 h with 20  $\mu\text{l}$  of

cytokeratin antibody diluted 1:500 in 1% BSA in PBS. Slides were washed three times with PBS for 5 min each during incubation. Each well was incubated with 20  $\mu$ l of the biotinylated secondary antibody for 15 min at RT.

Sections without primary antibodies served as negative controls. The sections were examined and scored at 400X magnification.

### **2.6.1.3 Vimentin**

The procedure was the same as that described for CD90 except for the incubation conditions of the primary and secondary antibodies. Each well was incubated at RT for 2 h with 20  $\mu$ l of vimentin antibody diluted 1:200 in 1% BSA in PBS. Slides were washed three times with PBS for 5 min each during incubation. Each well was incubated with 20  $\mu$ l of the biotinylated secondary antibody for 15 min at RT.

Sections without primary antibodies served as negative controls. The sections were examined and scored at 400X magnification.

### **2.6.1.4 SHH**

The procedure was the same as that described for CD90 except for the incubation conditions of the primary and secondary antibodies. Each well was incubated at RT for 2 h with 20  $\mu$ l of SHH antibody diluted 1:100 in 1% BSA in PBS. Slides were washed three times with PBS for 5 min each during incubation. Each well was incubated with 20  $\mu$ l of the biotinylated secondary antibody for 15 min at RT.

Sections without primary antibodies served as negative controls. The sections were examined and scored at 400X magnification.

## **2.6.2 Short tandem repeats (STR)**

### **2.6.2.1 Cell harvesting**

Cells (SCC4, SCC9, SCC15, SCC25, TU53, TU57 and TU63) were released with trypsin. After centrifugation at 1200 rpm for 5 min, the liquid was removed completely. Up to  $2 \times 10^5$  cells were suspended and aliquoted into 1.5-ml tubes, flash frozen in liquid nitrogen, then stored at  $-70\text{ }^\circ\text{C}$ .

### **2.6.2.2 Lysis of cells and tissues**

Cell pellets (SCC4, SCC9, SCC15, SCC25, TU53, TU57 and TU63) were suspended thoroughly in 200 ml of resuspension solution. 20 ml of proteinase K solution were added to the sample, followed by 200 ml of lysis solution C. Cells were vortexed thoroughly and incubated at  $70\text{ }^\circ\text{C}$  for 10 min.

180 ml of lysis solution T were added to tissues K53, K57 and K63, and primary cell cultures TU53, TU57 and TU63 respectively, followed by 20 ml of proteinase K solution. The samples were mixed by vortex then incubated at  $55\text{ }^\circ\text{C}$  for 2 h. 200 ml of lysis solution C were added to the samples. Then the samples were vortexed and incubated at  $70\text{ }^\circ\text{C}$  for 10 min.

### **2.6.2.3 DNA extraction**

500 ml of column preparation solution were added to each pre-assembled GenElute Miniprep Binding Column and centrifuged at  $12.000 \times g$  for 1 min. Flow through liquid was discarded. 200 ml of ethanol (95–100%) were added to the lysate and mixed thoroughly by vortex for 5–10 s. The entire contents of the tube were transferred to the treated binding column and centrifuged at  $6500 \times g$  for 1 min. The binding columns were placed in a new 2-mL collection tube. 500 mL of wash solution were added to the binding column and centrifuged for 1 min at  $6.500 \times g$ . The binding columns were placed in a new 2-ml collection tube. Another 500 ml of wash solution were added to the binding column and centrifuged for 3 min at maximum speed ( $12.000 \times g$ ) to dry the binding column. Finally, the binding columns were placed in new 2-ml collection tubes. 200 mL of the elution solution were pipetted directly into the center of the

binding column, incubated for 5 min at RT, and centrifuged for 1 min at  $6.500 \times g$  to elute the DNA.

#### 2.6.2.4 DNA measurement

The DNA samples were diluted in TE Buffer and absorbance was measured at 260 nm and 280 nm by spectrophotometric analysis. An absorbance of 1.0 at 260 nm corresponds to about 50 mg/ml of double stranded DNA. All DNA samples were prepared to a concentration of 5 ng/ $\mu$ l.

#### 2.6.2.5 PCR amplification of short tandem repeat (STR) regions

An AmpFISTR® Identifiler® Plus PCR Amplification Kit was used. The volume of reaction mix was calculated. Preparation details are shown in Table 2.

**Table 2:** Preparation of samples for PCR amplification of STR regions

DNA sample	To prepare...
AmpFISTR® Identifiler® Plus Master Mix	10.0 $\mu$ l
AmpFISTR® Identifiler® Plus Primer Set	5.0 $\mu$ l
Negative control	Add 10 $\mu$ l of MiniQ water
Positive control	Add 10 $\mu$ l of 9947A control DNA with the concentration of 0.1 ng/ $\mu$ l.
Test sample	Add 9.5 $\mu$ l MiniQ water and 0.5 $\mu$ l DNA samples with concentration of 5 ng/ $\mu$ l to the reaction mix.

The tubes were loaded into a thermal cycler and PCR amplification was performed under the thermal cycling conditions shown in Table 3.

**Table 3:** Thermal cycling conditions for PCR amplification

<b>Step1</b>	95 °C	11 min
<b>Step2</b>	94 °C	20 s
<b>Step3</b>	59 °C	3 min
<b>Step4</b>	Go to step 2	28 x
<b>Step5</b>	60 °C	10 min
<b>Step6</b>	4 °C	indefinitely

The whole PCR run lasted 2 h and 30 min.

### 2.6.2.6 Detection of STR amplicons

Samples for electrophoresis were prepared on the 3130 instrument immediately before loading. The volumes of Hi-Di™ Formamide and GeneScan™ 500 LIZ® Size Standard were calculated for preparation of the samples as shown in Table 4.

**Table 4:** STR amplicon detection components

<b>Reagent</b>	<b>Volume per reaction (μl)</b>
GeneScan 500 LIZ Size Standard	0.3
Hi-Di Formamide	11.7
PCR product (including the positive, negative samples and allelic ladder)	0.5 μl

The reaction plate was sealed with appropriate septa, and then briefly centrifuged to ensure that the contents of each well were collected at the bottom. The DNA within the reaction plate was denatured using a thermal cycler for 3 min at 95 °C. The plate was then immediately placed on ice for 3 min.

The 3130 Genetic Analyzer was managed following the manufacturer's instructions. Each electrophoresis run was started under the conditions shown in Table 5.

**Table 5:** Conditions for each electrophoresis run of the 3130 Genetic Analyzer.

<b>Index</b>	<b>Amount</b>
Injection voltage	3000 V
Injection time	10 s
Voltage number steps	40
Data delay time	1 s
Run time	1200 s



### **2.6.2.7 Data analysis and STR genotyping**

After electrophoresis, the Data Collection Software stored information for each sample and allelic ladder samples in an individual run, which was considered to be the valid standard controls while using GeneMapper® *ID* software for analyses and interpretation of the data.

## **2.7 Drug Study**

### **2.7.1 Preparation of working solutions**

Cilengitide and vismodegib were purchased from Selleck Chemicals (USA). DMSO was added to the inhibitor powder to prepare stock solution according to instructions. The dilution was calculated and then the stock solution was slowly added to the cell culture medium until the desired concentration, from 6.25  $\mu\text{M}$  to 100  $\mu\text{M}$ , was obtained. The solution was then mixed by vortex and stored at 4 °C.

### **2.7.2 MTT test**

A proliferation assay was conducted using an MTT Cell Proliferation Kit (Roche Applied Science, IA, USA). Cell lines (SCC4, SCC9, SCC15 and SCC25) and primary cell cultures (TU53, TU57 and TU63) were cultured with different concentrations of cilengitide from 6.25  $\mu\text{M}$  to 100  $\mu\text{M}$  by fold. The negative control groups were cells cultured in medium with DMSO, but without either drug. The blank control groups were medium with DMSO, but without either of the drugs or cells. Six parallel wells were provided. After a 72-h incubation period, 10  $\mu\text{l}$  of MTT labeling reagent was added to every well. Each microplate was incubated at 37 °C in a humidified atmosphere for 4 h, then 100  $\mu\text{l}$  solubilization solution was added to every well, and the microplate was incubated overnight. Absorbance at 550 nm was measured using an automatic microplate reader.

### **2.7.3 BrdU test**

Proliferation with cilengitide or vismodegib was measured using a BrdU Cell Proliferation Kit (Roche Applied Science, IA, USA). Cells were re-suspended with DMEM medium containing 10% fetal bovine serum and seeded onto 96-well plates at a density of  $3 \times 10^3$  cells/well. The final volume of each well was 100  $\mu$ l. After being cultured at 37 °C for 24 h, 100  $\mu$ l of cilengitide or vismodegib working solution were added at a final concentration of 100, 50, 25, 12.5 or 6.25  $\mu$ M. The negative control groups comprised cells with 100  $\mu$ l medium without either drug, and the blank control comprised only medium. Six parallel wells were provided for each treatment 72 h after exposure.

10  $\mu$ l BrdU labeling solution was added to each well and the plates were kept overnight in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. After removing the labeling solution, 200  $\mu$ l/well FixDenat solution was added and incubated for 30 min at RT, followed by thorough removal of the solution. 100  $\mu$ l/well of anti-BrdU-POD working solution was then added. After incubation for 2 h at RT, the antibody conjugate was removed by rinsing the plate three times with washing buffer made in PBS. Subsequently, 100  $\mu$ l/well substrate solution was added and incubated at RT for 15 min until color development was sufficient for photometric detection. Prior to the measurement of all samples, 50  $\mu$ l 1 M H<sub>2</sub>SO<sub>4</sub> were added to the cells to stop the reaction. Cells cultured in serum-free culture medium served as controls. Finally, absorbance was measured at 450 nm using the microplate reader. The absorbance values of each well were saved and analyzed.

### **2.7.4 Quantitative Real-Time PCR**

#### **2.7.4.1 Drug treatment**

Quantitative real-time PCR was performed to evaluate changes in gene expression of ITGAV, ITGBV3, and ITGBV5 after treatment with cilengitide, and of SHH, PTCH1, SMO, SUFU and GLI1 after incubation with vismodegib.

Cell lines SCC4, SCC9, SCC15, SCC25 and primary cell cultures TU53, TU57, TU63 were re-suspended with DMEM medium containing 10% fetal bovine serum and seeded onto 6-well plates at a density of  $3 \times 10^4$  cells/ml. The final volume of each well was 3 ml. After being cultured in an incubator for 24 h, the medium was changed to the working solution with cilengitide as the experimental group at a final concentration of 50  $\mu$ M or 100  $\mu$ M. For the control group, the medium was changed to the working solution without the drug, but with

DMSO as the solvent. Three parallel wells were provided for each group. The cells were harvested 72 h after treatment.

#### **2.7.4.2 RNA extraction**

An RNeasy Mini Kit was used to extract total RNA. The medium was aspirated, and the cells were washed once with PBS. The cells were trypsinized from the well, and centrifuged at 300 g for 5 min. The supernatant was completely aspirated, and 350  $\mu$ l of RLT Plus buffer were added and mixed by vortex for 1 min. The lysates were pipetted directly into a QIA shredder spin column placed in a 2-ml collection tube, and centrifuged for 2 min at maximum speed. The homogenized lysate was transferred to a DNA Eliminator spin column placed in a 2 ml collection tube and centrifuged for 30 s at 8000 g, and the flow-through was saved. 350  $\mu$ l of 70% ethanol were added and mixed. 700  $\mu$ l of the sample were transferred to an RNeasy spin column in a 2-ml collection tube. Samples were centrifuged for 15 s at 8000 g and the flow-through was discarded. 700  $\mu$ l of RW1 buffer were added and centrifuged for 15 s at 8000 g to wash the spin column membrane. The flow-through was discarded. 500  $\mu$ l of RPE buffer were added and centrifuged for 15 s at 8000 g ( $\geq 10,000$  rpm) to wash the spin column membrane. The flow-through was discarded and the membrane washed again. The RNeasy spin columns were placed in a new 1.5-ml collection tube. 30  $\mu$ l RNase-free water were added and centrifuged for 1 min at 8000 g to elute the RNA.

The RNA was diluted in 10 mM Tris·HCl, pH 7.0 and absorbance was measured at 260 nm and 280 nm by spectrophotometric analysis. An absorbance of 1.0 at 260 nm corresponds to about 44  $\mu$ g/ml of RNA.

#### **2.7.4.3 Reverse Transcription**

According to the results of RNA measurement, the amount of 1  $\mu$ g of template RNA was calculated for each sample. Genomic DNA elimination reaction was prepared on ice according to Table 6.

**Table 6:** Genomic DNA elimination reaction components

<b>Component</b>	<b>Volume</b>
gDNA wipeout buffer, 7x	2 $\mu$ l

Template RNA	Equivalent to 1µg
RNase-free water	Variable
Total amount	14 µl

Samples were incubated for 2 min at 42 °C then placed immediately on ice. The reverse-transcription master mix (Table 7) was prepared on ice.

**Table 7:** Reverse-transcription reaction components

Component	Volume
Quantiscript reverse transcriptase	1 µl
Quantiscript RT Buffer, 5x	4 µl
RT Primer Mix	1 µl
Entire genomic DNA elimination reaction	14 µl
Total volume	20 µl

Samples were incubated at 42 °C for 15 min, and then incubated for 3 min at 95 °C to inactivate Quantiscript reverse transcriptase. Reverse-transcription reaction components were stored at -20 °C.

#### 2.7.4.4 Quantitative Real-Time PCR

A reaction mix was prepared in accordance with the QuantiTect-SYBR-Green-PCR-Handbook (Table 8). Capillary cyclers were used for two-step RT-PCR.

**Table 8:** Quantitative Real-Time PCR reaction mix components

Component	Volume
2x QuantiTect SYBR Green PCR Master Mix	10 µl
10x QuantiTect Primer Assay	2 µl
Template cDNA	1 µl
RNase-free water	7 µl
Total volume	20 µl

The reaction solution was mixed thoroughly, and appropriate volumes were dispensed into PCR capillaries. Template cDNA was added to the individual PCR capillaries containing the

reaction mix. Negative controls contained reaction mix without template cDNA. PCR capillaries were placed in the real-time Light Cycler, which was programmed as shown in Table 9.

**Table 9:** Cycling conditions for two-step qPCR using capillary cyclers

Step	Time	Temperature
PCR initial activation step	15 min	95 °C
Denaturation	15 s	94 °C
Annealing	20 s	55 °C
Extension	20 s	72 °C
Number of cycles	40	

All primers (Table 10) were obtained from QIAGEN. The results were analyzed using the Light Cycler Software, version 3. The final PCR products were evaluated by agarose gel electrophoresis to verify the size of the cDNA products by comparison with a low range DNA ladder of 25-500 bp. Thus, 10- $\mu$ l aliquots of each sample were electrophoresed on a 2.0% agarose gel in TBE buffer stained with ethidium bromide and photographed using the BioDocAnalyze System.

**Table 10:** List of all primers for RT-PCR

Gene (official name)	Gene (abbreviated)	Primer Size	Forward primer	Reverse primer
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	95 bp	ACAACTTTGGT ATCGTGGAAGG	GCCATCACGCC ACAGTTTC
Integrin, alpha V	ITGAV	125 bp	GCTGTCGGAGA TTTCAATGGT	TCTGCTCGCCA GTAAAATTGT
Integrin, beta 3	ITGB3	116 bp	GTGACCTGAAG GAGAATCTGC	CCGGAGTGCAA TCCTCTGG

Integrin, beta 5	ITGB5	61 bp	AACTCGCGGAG GAGATGAG	AACTCGCGGAG GAGATGAG
Sonic hedgehog	SHH	136 bp	CTCGCTGCTG GTATGCTCG	ATCGCTCGGA GTTTCTGGAG A
Patched 1	PTCH1	119 bp	ACTTCAAGGG GTACGAGTAT GT	TGCGACACTC TGATGAACCA C
Smoothened, frizzled class receptor	SMO	89 bp	GTGAGCGCCT CATCGAAGT	AGCGACTGGA CGTAGGTGA
Suppressor of fused homolog	SUFU	92 bp	AGACCCCTTG GACTATGTTA GC	CGAAGCTGAT GTAGTGCCAG T
GLI family zinc finger 1	GLI1	148 bp	AGCGTGAGCC TGAATCTGTG	CAGCATGTAC TGGGCTTTGA A

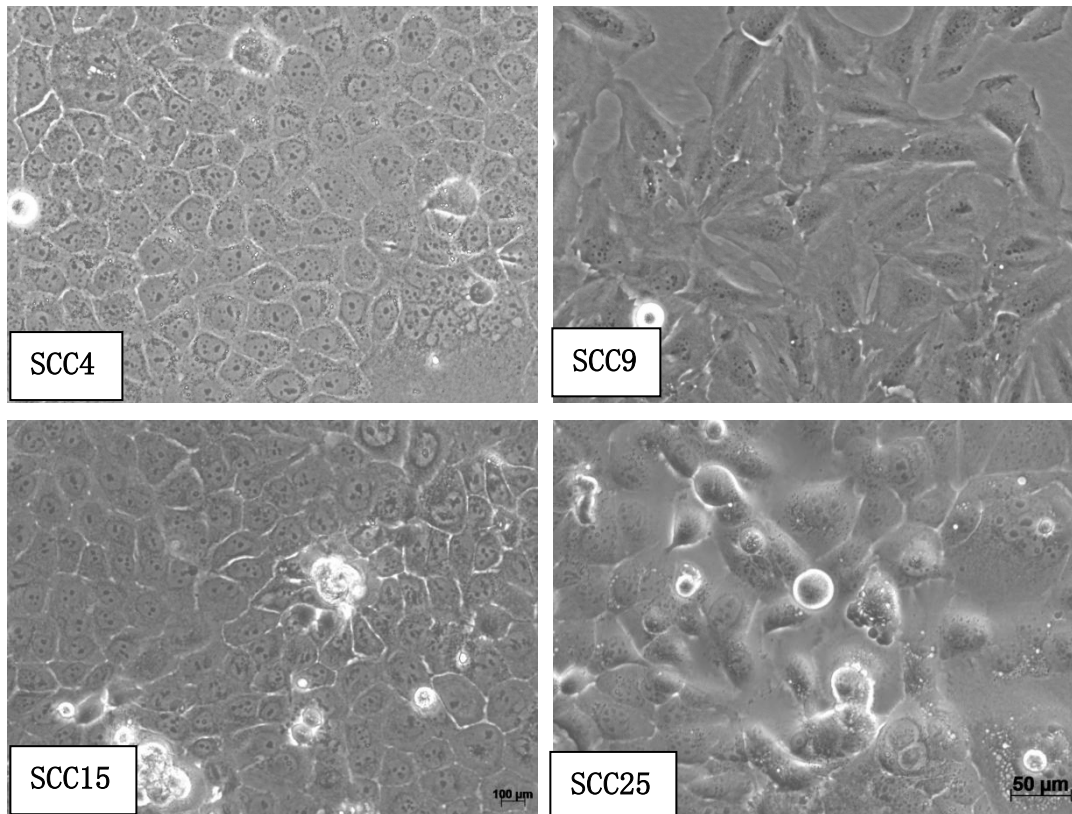
## 2.8 Statistical analysis

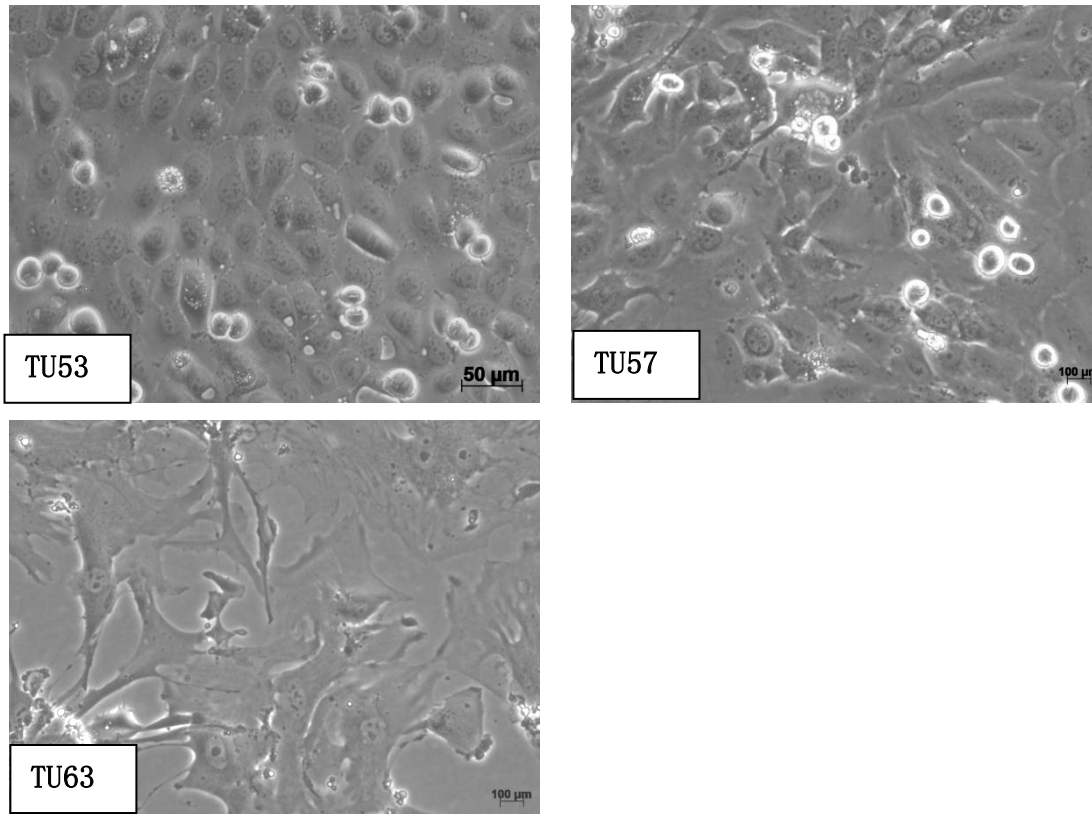
All experiments were performed in triplicate. The absorbance values of the MTT and BrdU tests are presented as means  $\pm$  SD. Data were analyzed by one-way analysis of variance for comparison with controls. If significant F-statistics from analysis of variance existed, this test was followed by a Dunnett post-hoc multiple comparison among all groups. A P-value of  $<0.05$  was considered statistically significant.

## 3. Results

### 3.1 SCC morphology

Cells began to adhere to the disk after 2 to 3 h. They exhibited first as small, round, non-uniform sized dots, gradually extending into short or long spindles and finally forming polygons and an epithelium-like morphology within 24 h. All cells showed a homogeneous epithelium-like shape (Fig. 4). These cells reached 80-90% confluence every 4 to 5 days.





**Figure 4:** Morphology of SCCs under the microscope at a magnification of 400X. Adherent cells were polygonal epithelial-like and cobblestone-like. Some round cells with multiple nuclei are visible, in which the nucleoli are clear and unequal in size; there were up to five or six nucleoli present.

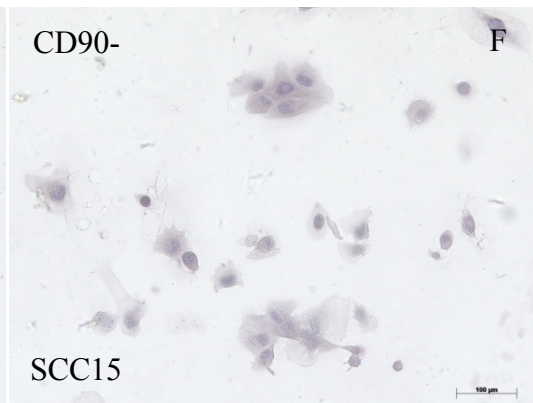
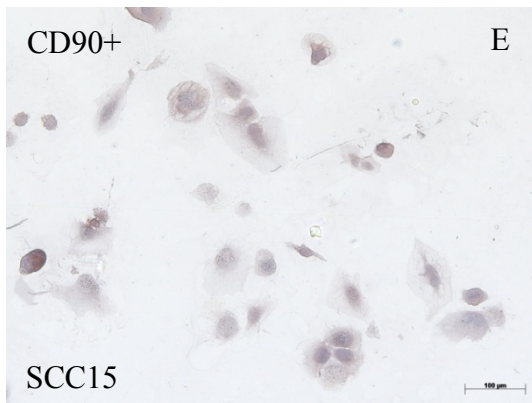
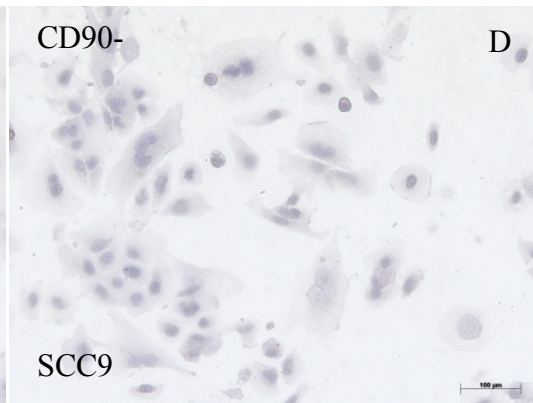
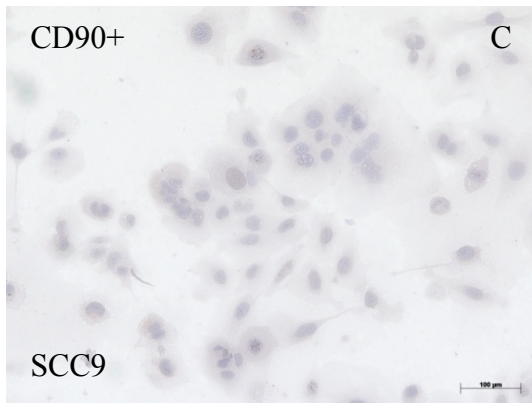
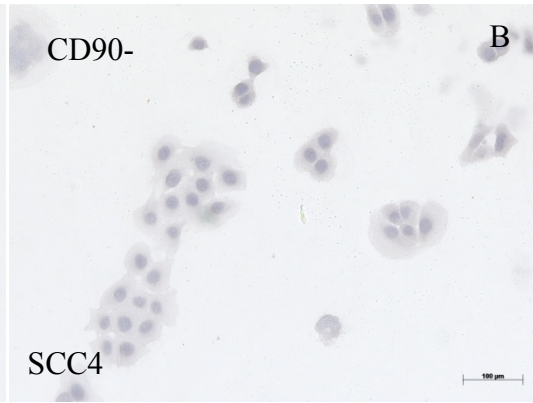
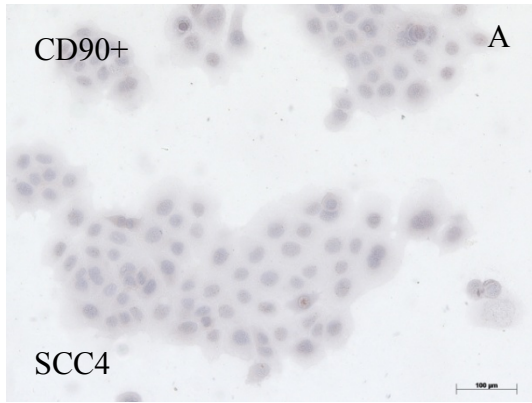
## 3.2 Immunocytochemical staining

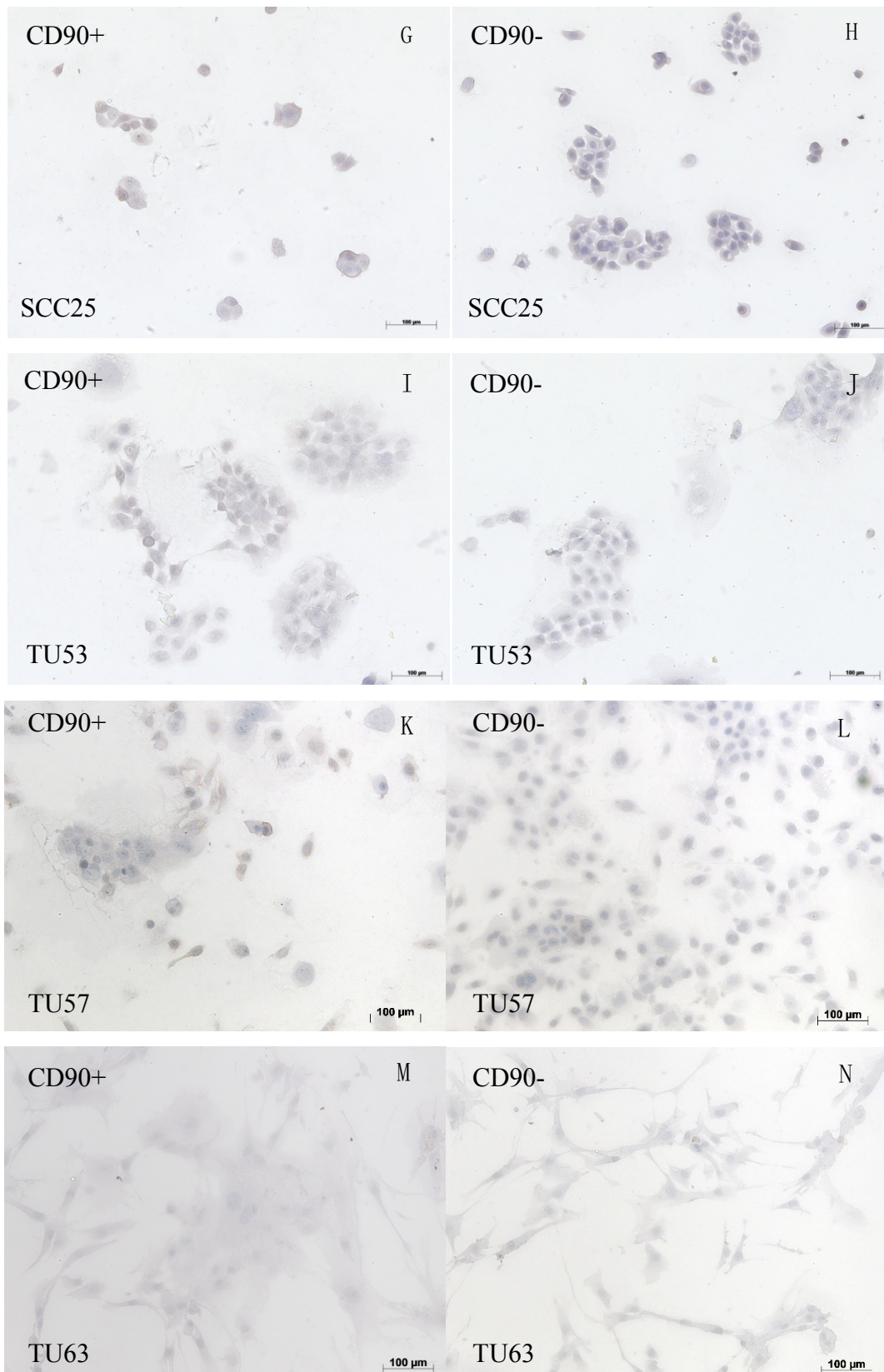
To identify the biological characteristics of the primary cell cultures and cell lines, four kinds of standard immunocytochemical staining were performed.

### 3.2.1 CD90 staining

CD90 is a cell surface protein with a single V-like immunoglobulin domain. CD90 was originally discovered as a thymocyte antigen, and used as a marker for a variety of stem cells, axonal processes of mature neurons, and a fraction of fibroblasts and myofibroblasts. All cells expressed negative CD90 (Figure. 5).





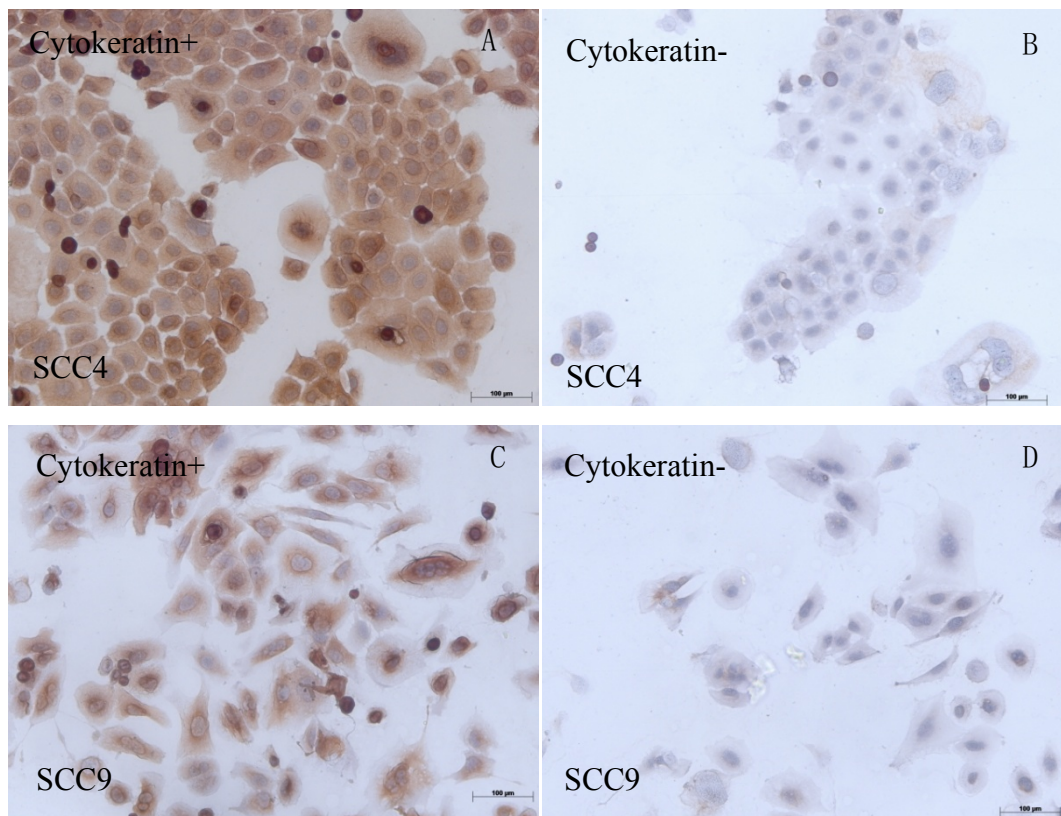


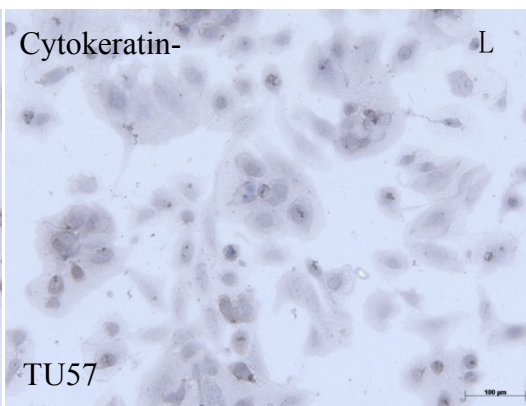
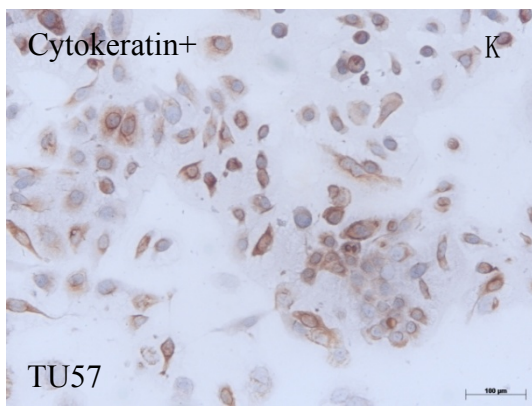
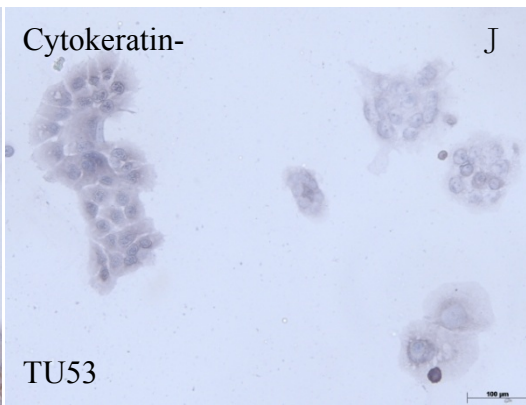
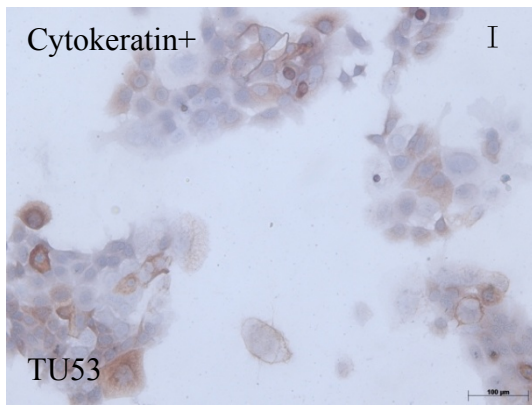
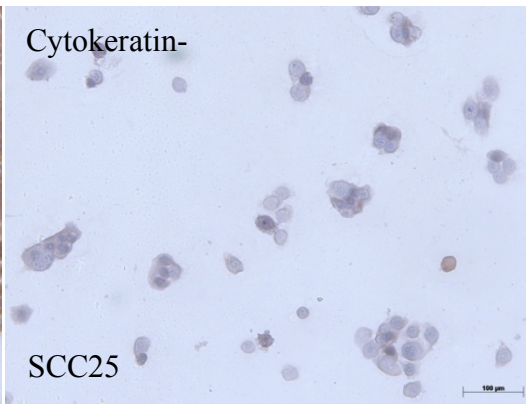
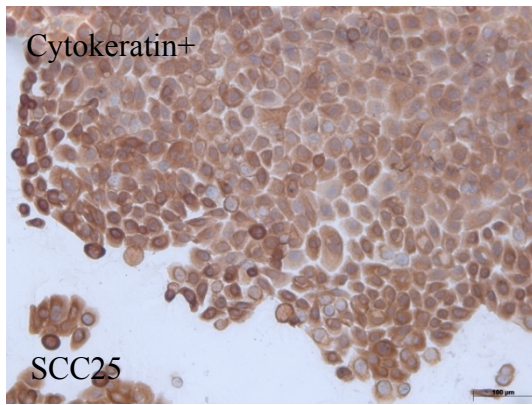
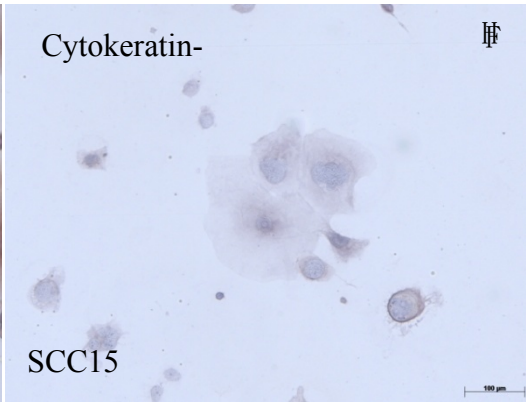
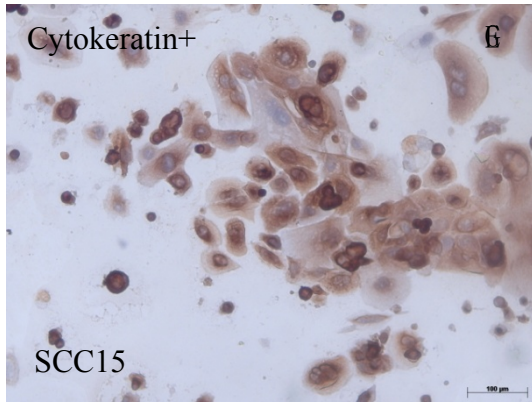
**Figure 5:** Immunocytochemical staining of CD90 in HNSCC cells. Each well was incubated at 4 °C overnight with 20 µl of CD90 antibody diluted 1:100 in 1% BSA in PBS, and with 20 ml biotinylated secondary antibody for 30 min at RT. Sections without primary antibodies

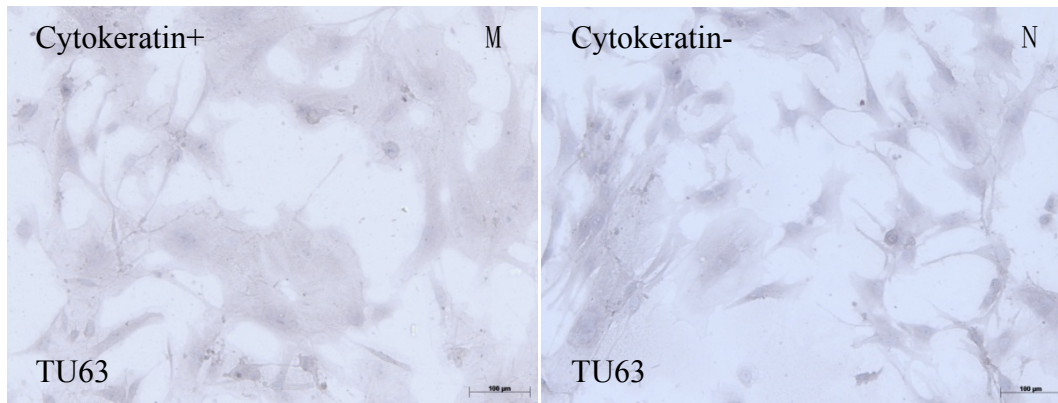
that served as negative controls are also shown. The sections were examined and scored using a microscope at 400X magnification. Expression of CD90 in cell lines (A, C, E, G) and primary cell cultures (I, K, M) was negative.

### 3.2.1 Cytokeratin staining

Cytokeratin is characteristic of epithelial cells. Anti-cytokeratin is a broadly reactive reagent, which recognizes epitopes present in most human epithelial tissues. Both cell lines and primary cell cultures showed strong expression of cytokeratin (Fig. 6).



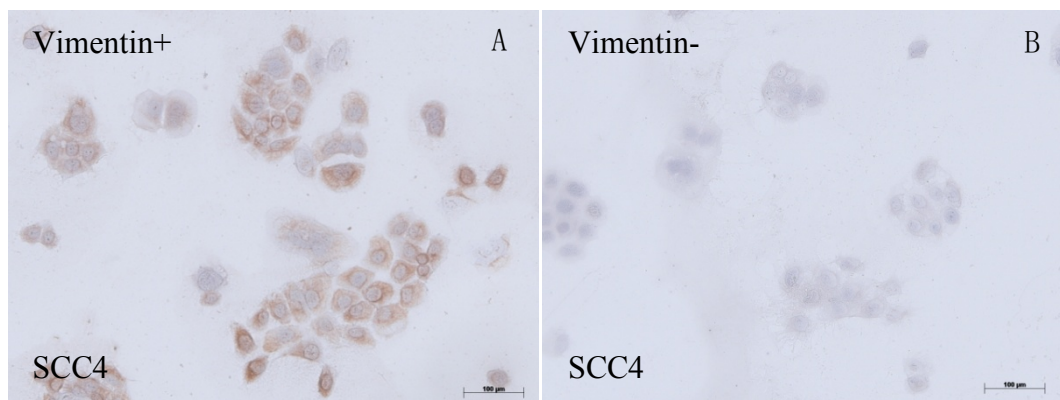


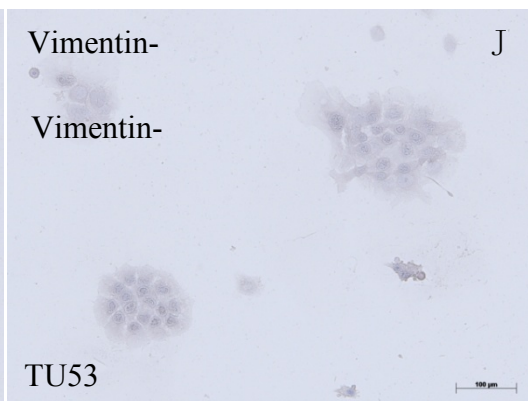
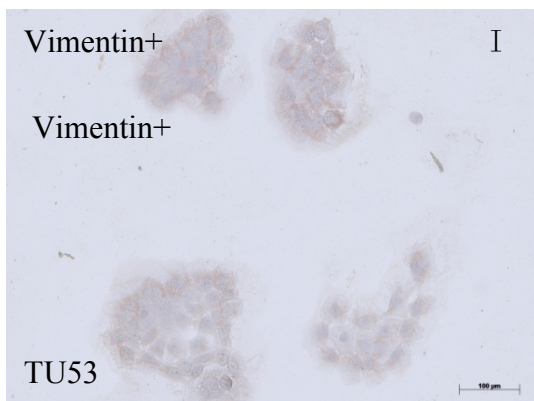
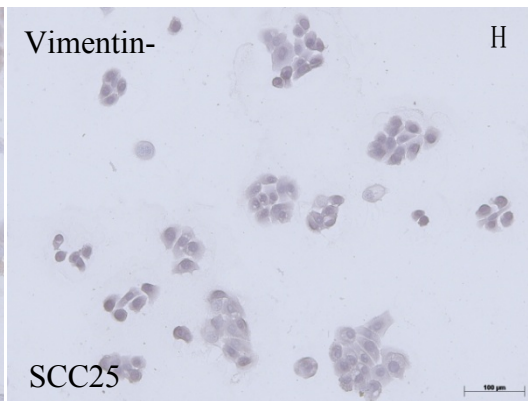
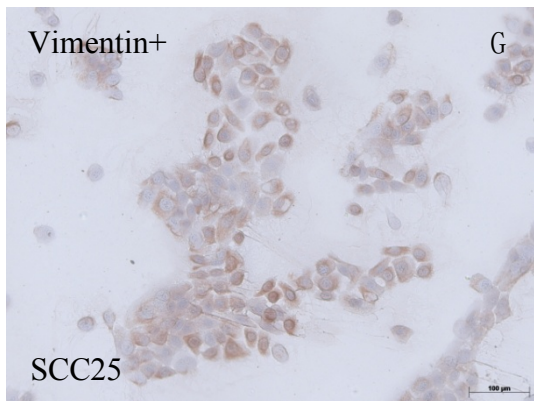
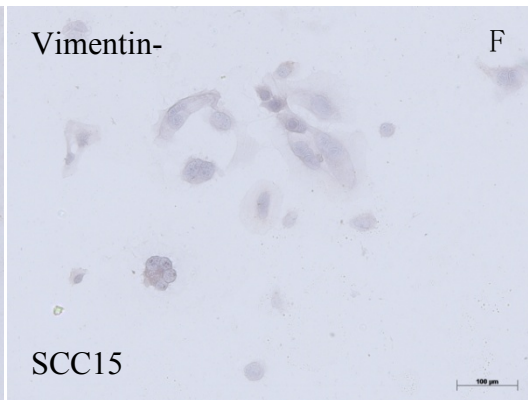
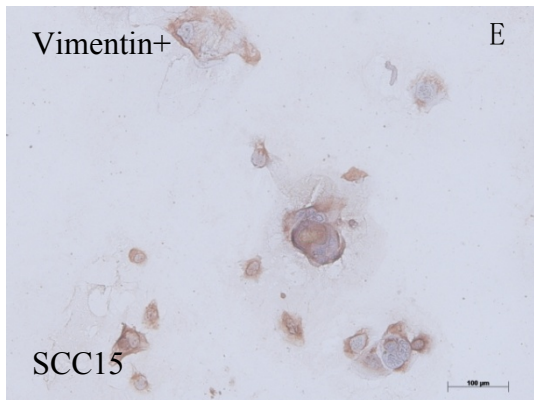
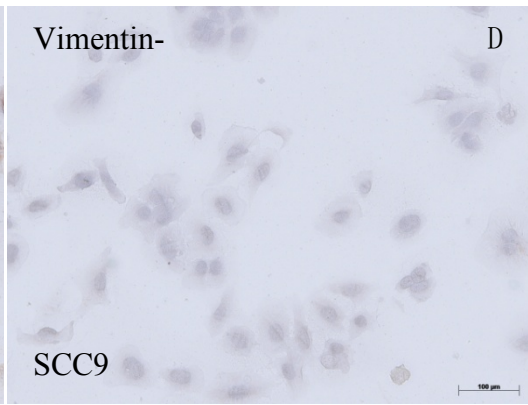
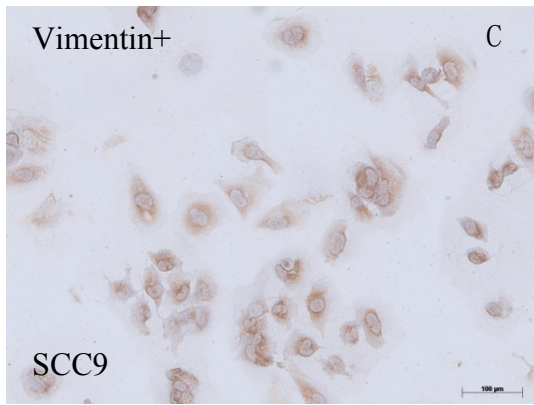


**Figure 6:** Immunocytochemical staining of cytokeratin in HNSCC cells. Each well was incubated at RT for 1 h with 20 µl of cytokeratin antibody diluted 1:500 in 1% BSA in PBS, and with 20 µl of the biotinylated secondary antibody for 15 min at RT. Sections without primary antibodies that served as negative controls are also shown. The sections were examined and scored using a microscope at 400X magnification. Cell lines (A, C, E, G) expressed strong positive cytokeratin. Primary cell cultures (I, K, M) showed modest expression of cytokeratin.

### 3.2.2. Vimentin staining

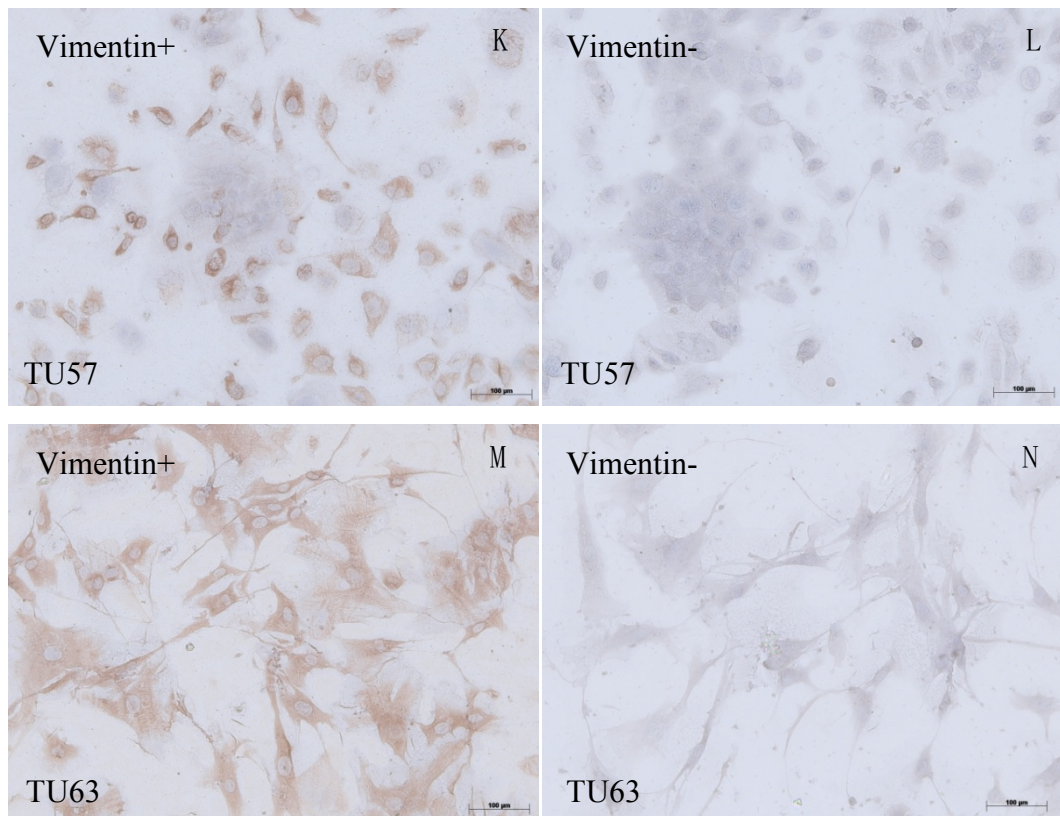
Vimentin is the major cytoskeletal component of mesenchymal cells. It is often used as a marker of mesenchymally derived cells and has been used as a sarcoma tumor marker to identify mesenchyme. All cells showed moderate expression of vimentin (Fig. 7).





TU53

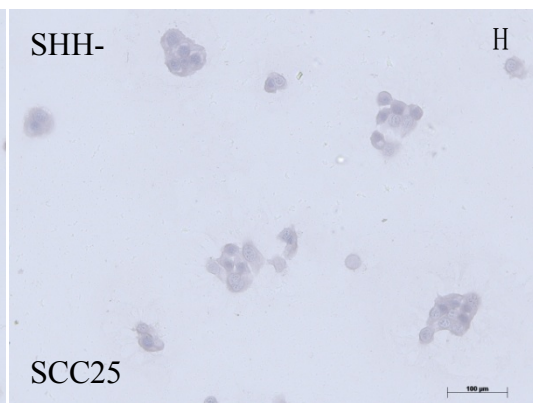
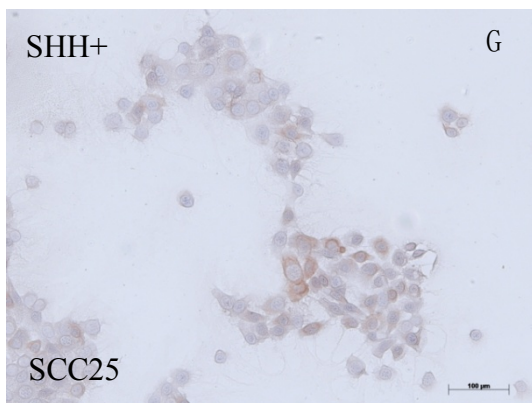
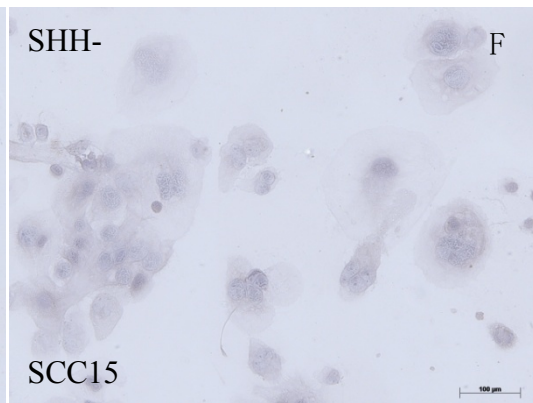
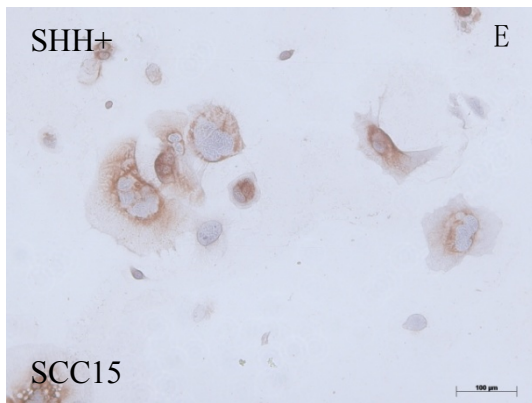
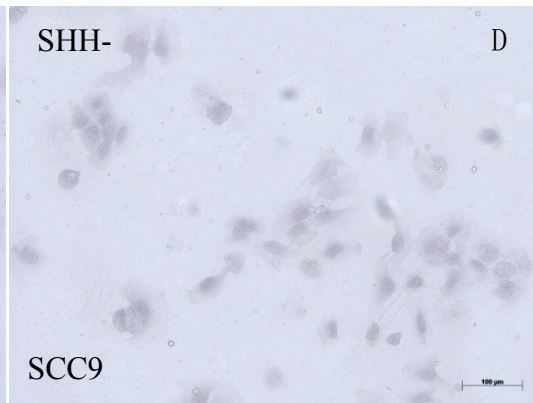
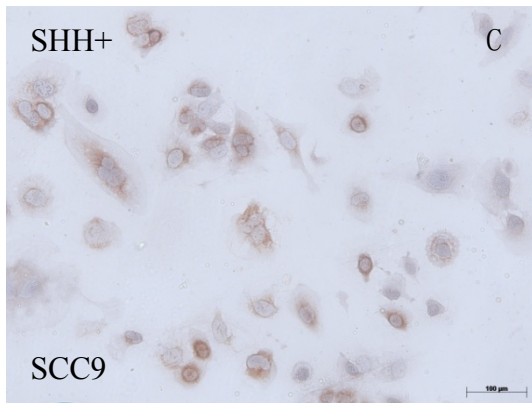
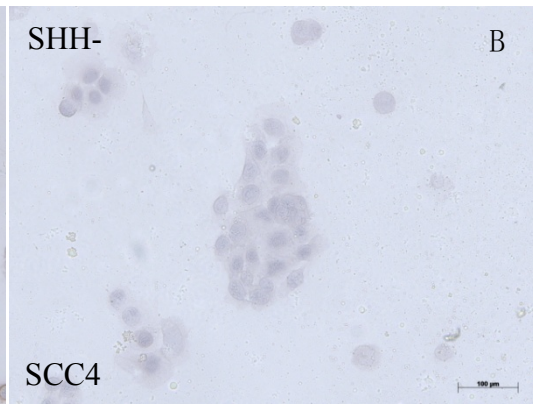
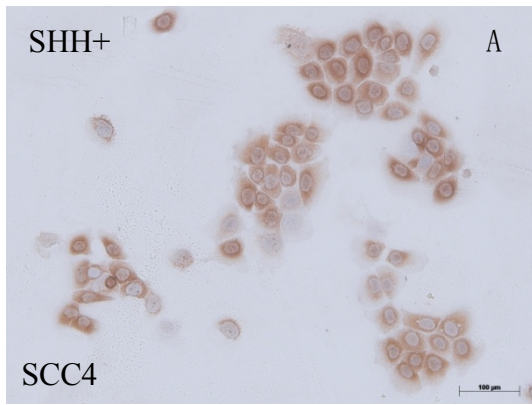
TU53



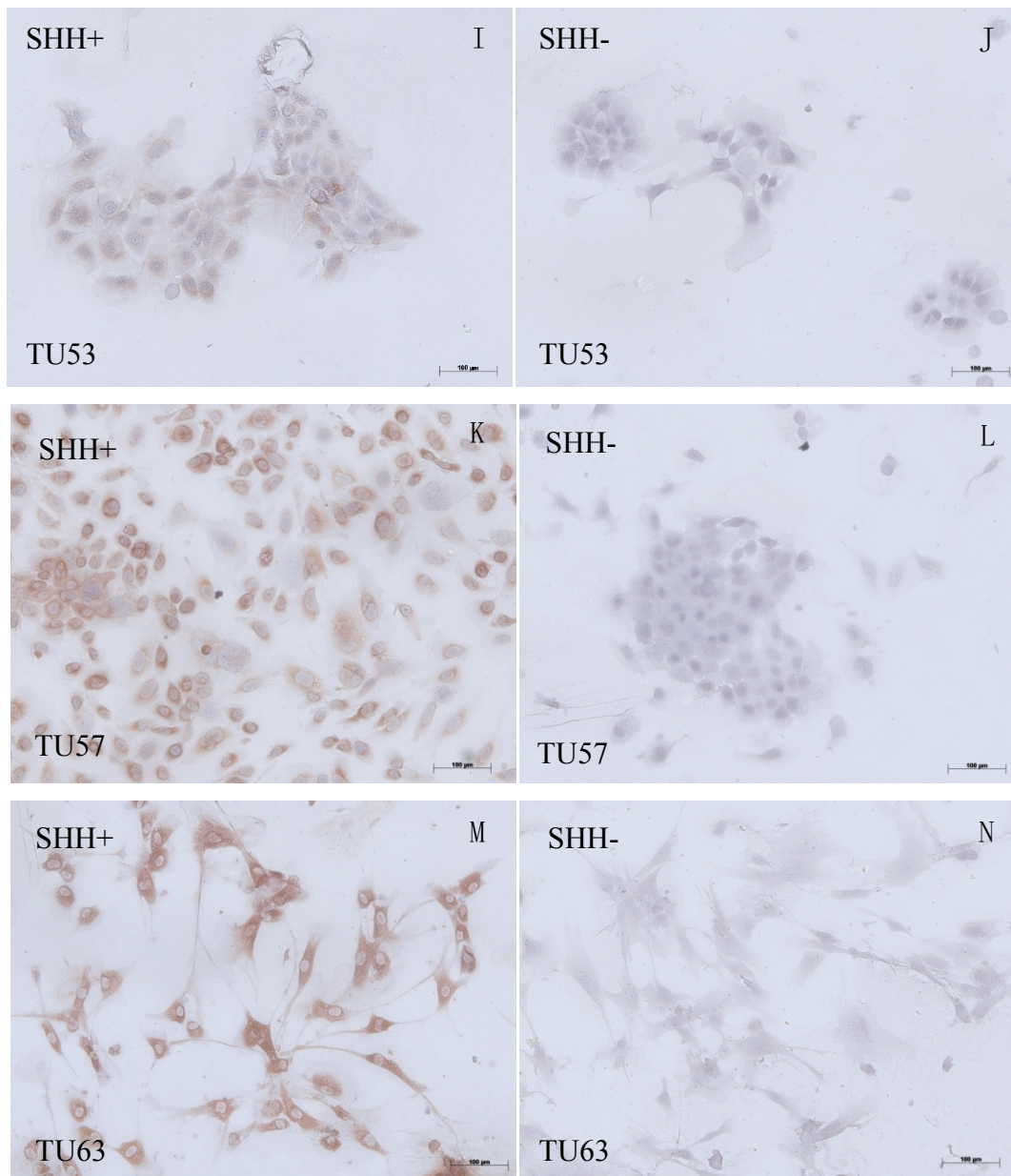
**Figure 7:** Immunocytochemical staining of vimentin in HNSCC cells. Each well was incubated at RT for 2 h with 20 µl of vimentin antibody diluted 1:200 in 1% BSA in PBS, and with 20 µl of the biotinylated secondary antibody for 15 min at RT. Sections without primary antibodies that served as negative controls are also shown. The sections were examined and scored using a microscope at 400X magnification. Cell lines (A, C, E, G) and primary cell cultures (I, K, M) showed modest expression of vimentin.

### 3.2.3 SHH staining

Sonic hedgehog (SHH) is a protein that controls cell division of adult stem cells and has been implicated in the development of some cancers, such as HNSCC, small cell lung cancer, pancreatic carcinoma, prostate cancer and gastric cancer. All cells expressed SHH (Fig. 8).







**Figure 8:** Immunocytochemical staining of SHH in HNSCC cells. Each well was incubated at RT for 2 h with 20  $\mu$ l of SHH antibody 1:100 diluted in 1% BSA in PBS, and with 20  $\mu$ l of the biotinylated secondary antibody for 15 min at RT. Sections without primary antibodies that served as negative controls are also shown. The sections were examined and scored using a microscope at 400X magnification. Cell lines (A, C, E, G) and primary cell cultures (I, K, M) showed modest expression of SHH.

### 3.3 STR

Sixteen core STR loci (Table 11) had been identified for estimating the homology of individual tissue and primary cell culture genetic profiles.

The DNA profiling showed that K53 and TU53, K57 and TU57, K63 and TU 63 are all homologous.

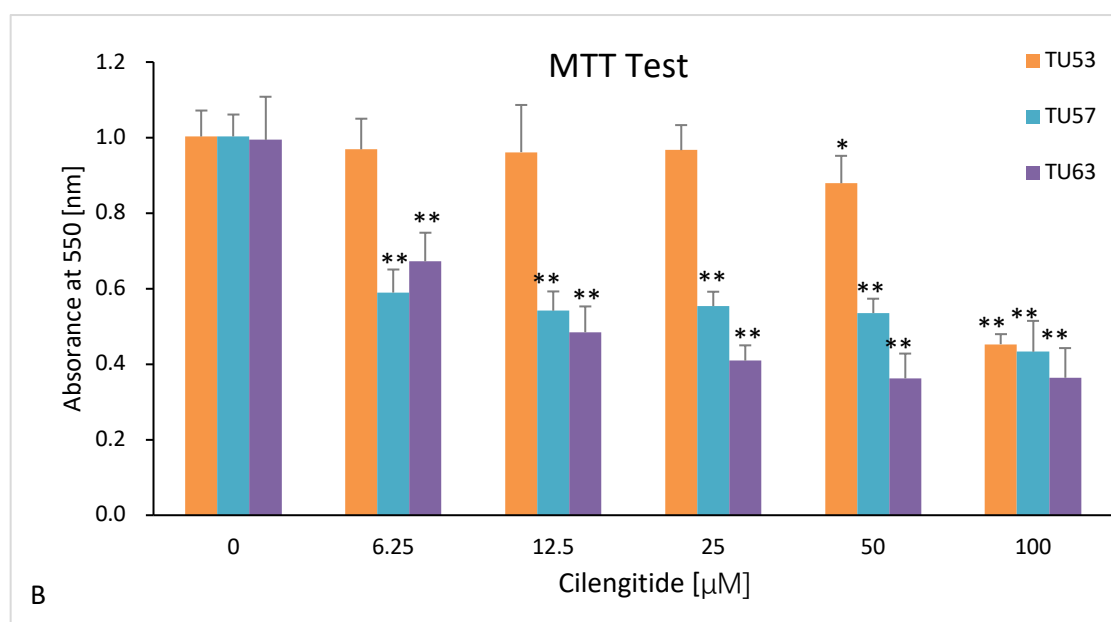
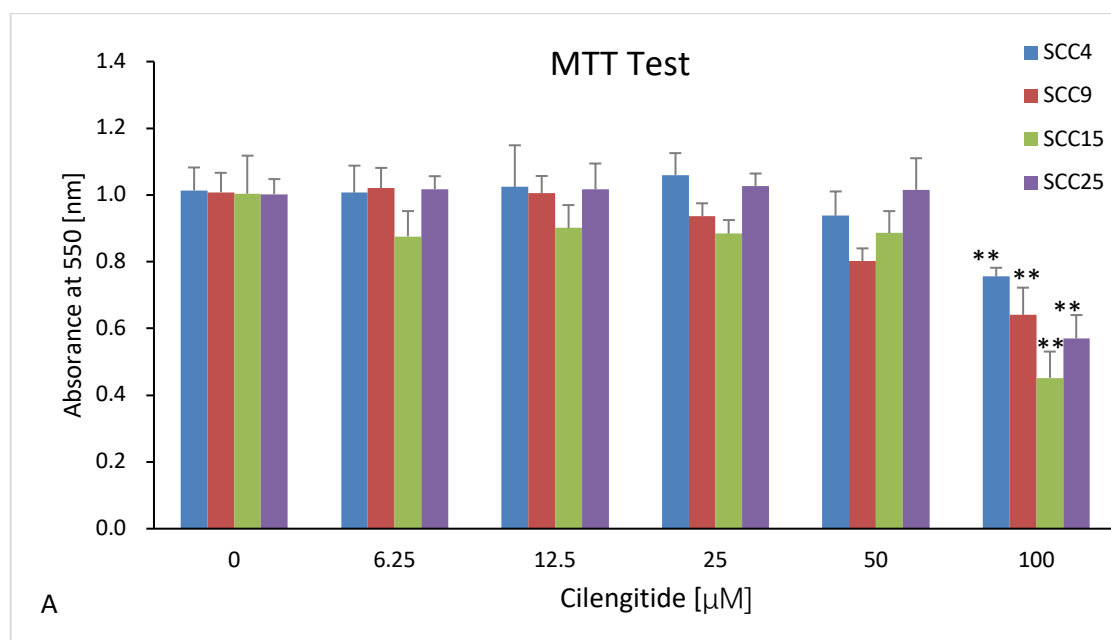
**Table 11:** Sixteen core STR loci of individual tissue and primary cell culture samples

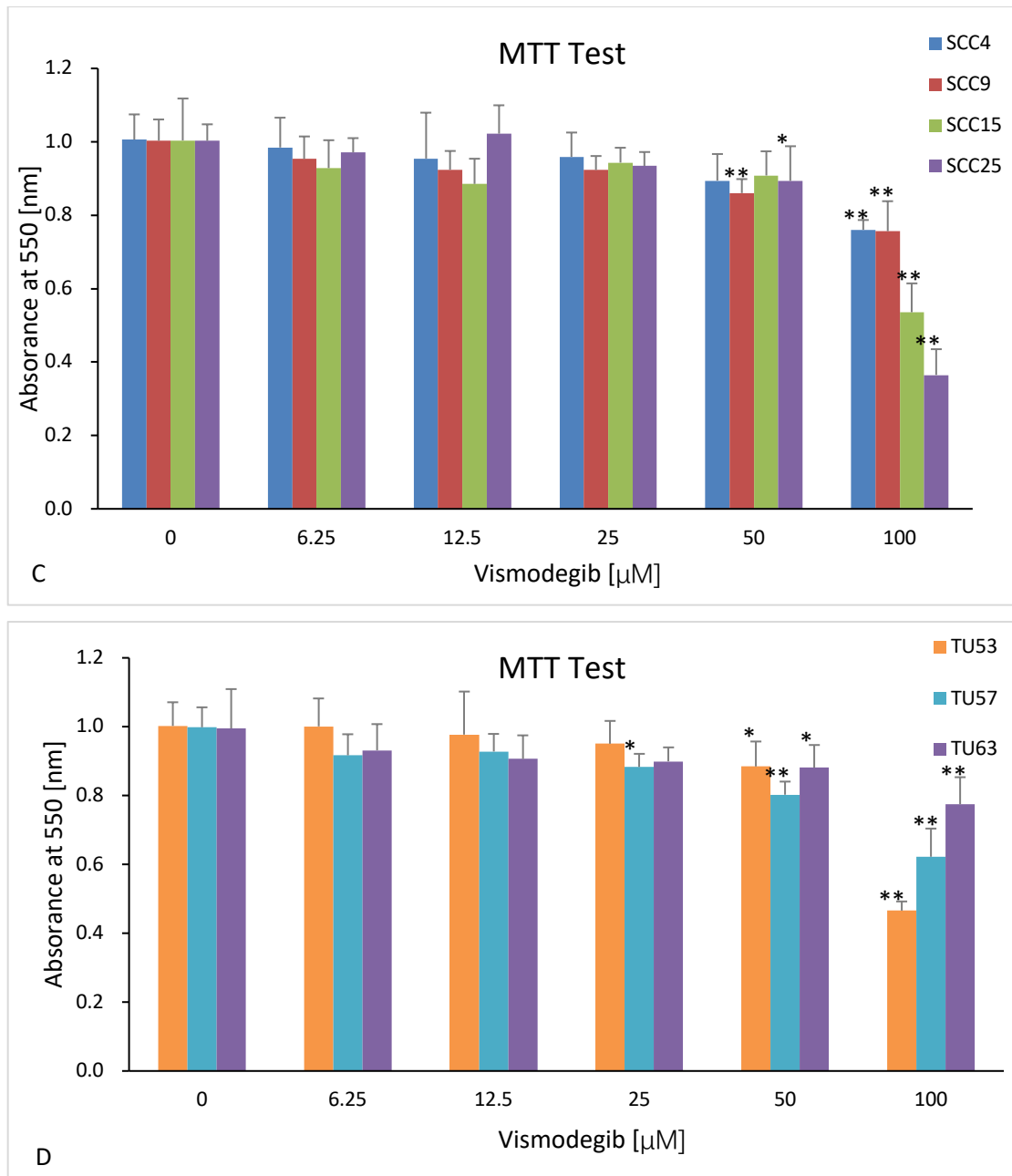
	<i>D8S1179</i>	<i>D21S11</i>	<i>D7S820</i>	<i>CSF1PO</i>	<i>D3S1358</i>	<i>TH01</i>	<i>D13S317</i>	<i>D16S539</i>
<b>K53</b>	3; 15	8.2; 33	1; 11	OL; OL	7; 17	3; 9.3	13	1; 11
<b>TU53</b>	OL; 15	8,2; 33	1; 11	OL; OL	7; 17	3; 9,3	13	1; 11
<b>K57</b>	0; 15	9.2; 32	OL; 12	OL; 10.2	6; 16	9.3	11	0; 11
<b>TU57</b>	0; 15	9.2; 32	0; 12	OL; OL	6; 16	9.3	11	0; 11
<b>K63</b>	3; 15	2; 32	12	OL; 10.2	5; 18	7	0; 14	0; 12
<b>TU63</b>	3; 15	2; 32	OL; 12	OL; 10.2	5; 18	7	0; 14	0; 12
	<i>D2S1338</i>	<i>D19S433</i>	<i>WA</i>	<i>OPX</i>	<i>D18S51</i>	....	<i>D5S818</i>	<i>GA</i>
<b>K53</b>	7; 17	3; 14.2	6; 16	OL; OL	4; 18.2	X	OL; OL	2; 26
<b>TU53</b>	7; 17	3; 14,2	6; 16	OL; OL	8,2; 18,2	X	OL; OL	OL; OL
<b>K57</b>	OL; OL	3; 14.2	5; 18	OL; OL	3; 21.2; 22.2	X	OL; OL	OL; 25
<b>TU57</b>	7; 17	3; 14.2	5; 18	9	OL; 21.2; 22.2	X	OL; OL	1; 21; 25
<b>K63</b>	4; 25	2; 12.2	9; 19	OL; OL	2; 16.2	Y	OL; OL	3; 23
<b>TU63</b>	4; 25	2; 12.2	9; 19	OL; OL	2; 16.2	Y	OL; OL	3; 23

## 3.4 Drug tests

### 3.4.1 MTT test

Proliferation of cell lines and primary cell cultures was suppressed by cilengitide or vismodegib in a dose dependent manner. Primary cell cultures showed more significant suppression than HNSCC cell lines. In primary cell cultures, the same concentration of drug could have a stronger inhibition effect (Fig. 9).

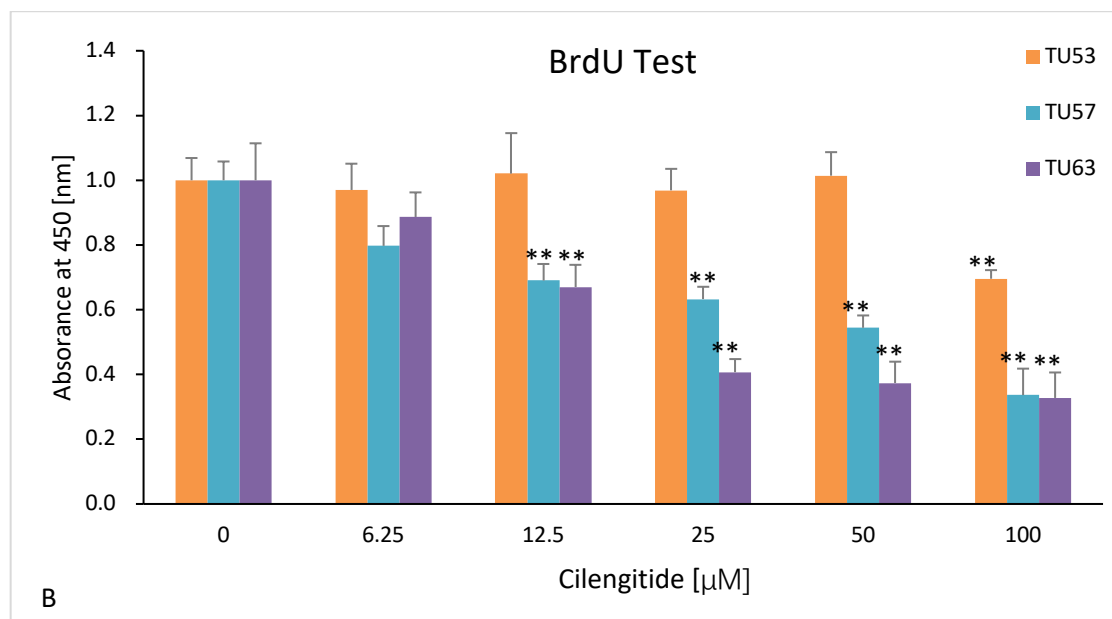
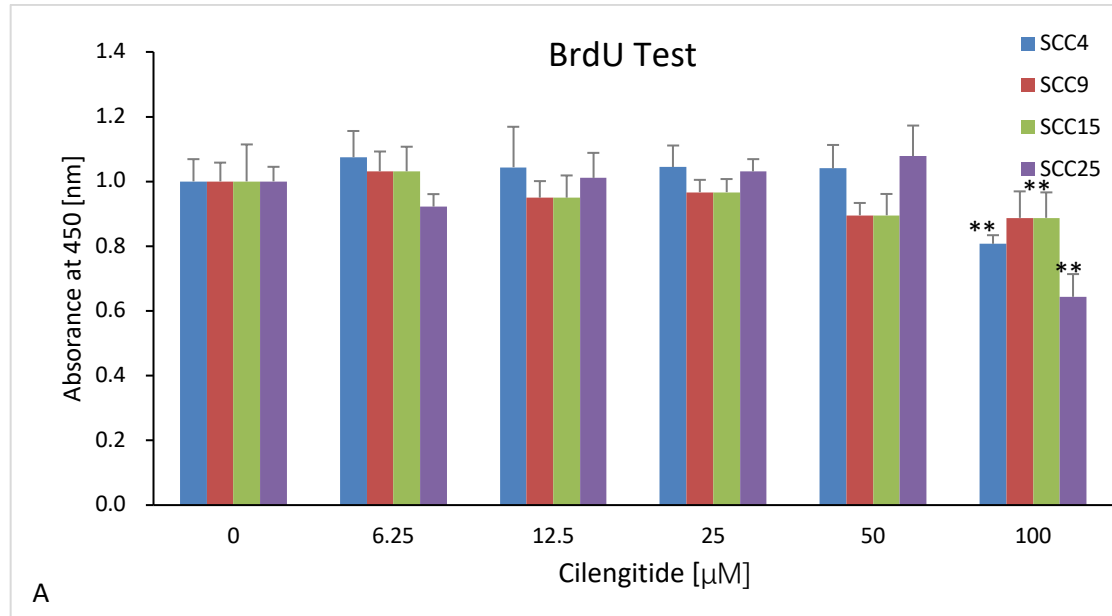


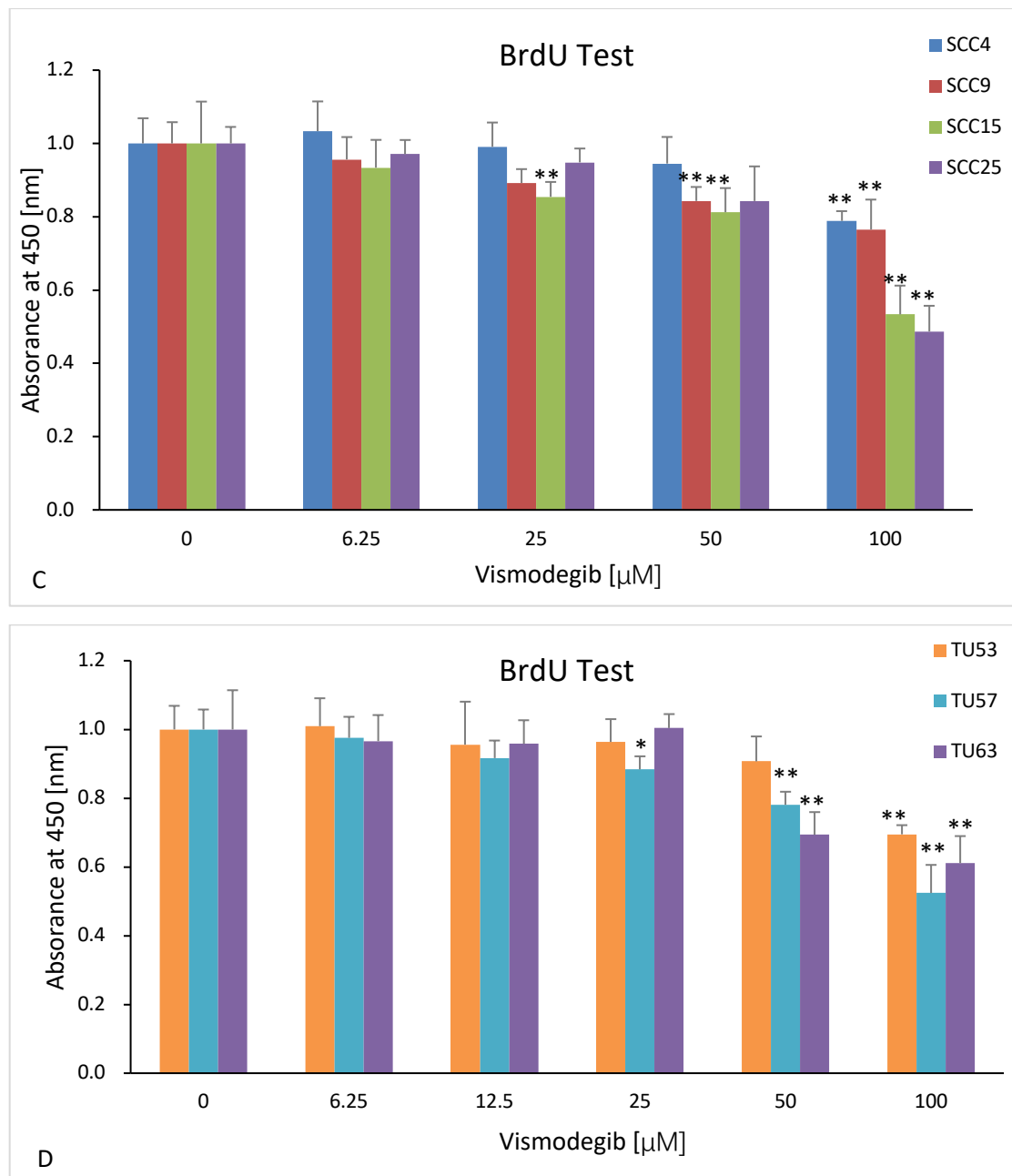


**Figure 9:** Cilengitide or vismodegib suppressed HNSCC cell proliferation. Cells were plated at a density of  $1 \times 10^5$  cells per well in a 96-well plate and were allowed to attach before incubation in medium containing cilengitide (A and B) or vismodegib (C and D) for 72 h. Absorbance at 550 nm was assessed with MTT tests. Mean  $\pm$  SEM (n=8). \*  $P < 0.05$ , \*\*  $P < 0.01$  (compared to the control). Proliferation of cell lines and primary cell cultures was suppressed by cilengitide (A and B) or vismodegib (C and D) in a dose dependent manner.

### 3.4.2 BrdU test

Proliferation of cell lines and primary cell cultures was suppressed by cilengitide or vismodegib. Primary cell cultures expressed more significant suppression of growth than HNSCC cell lines, especially with cilengitide (Fig. 10).



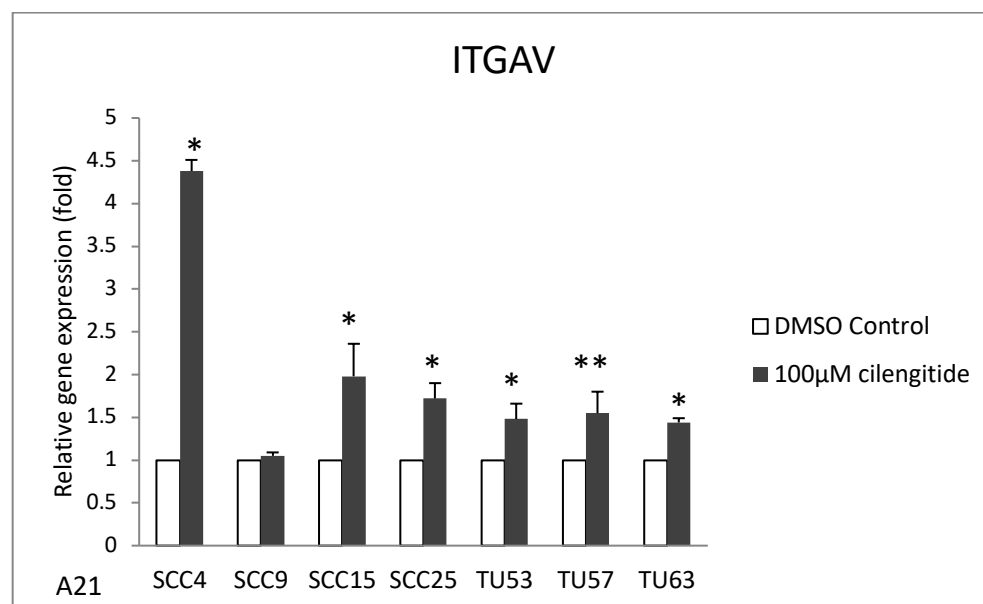
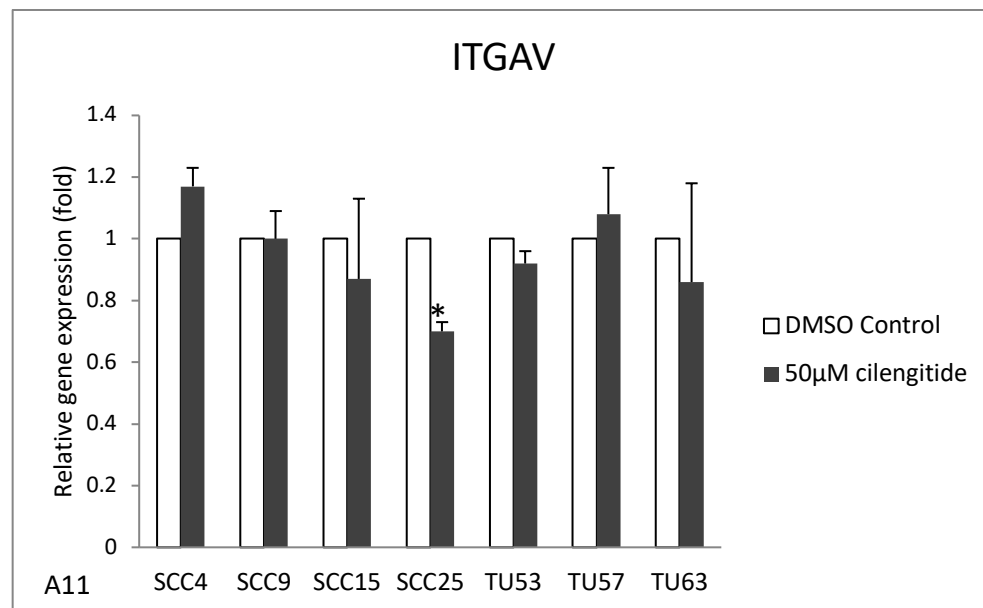


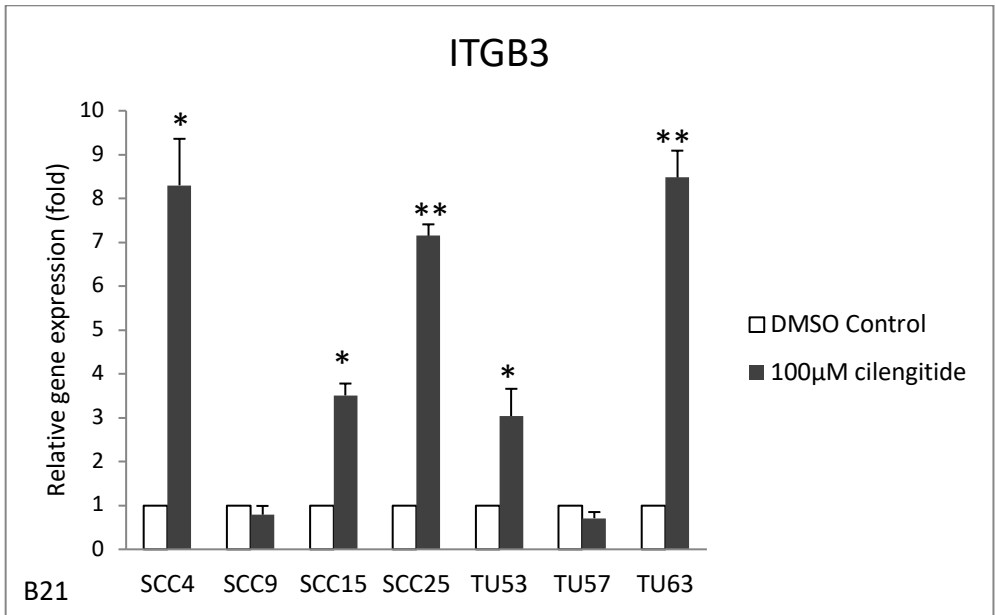
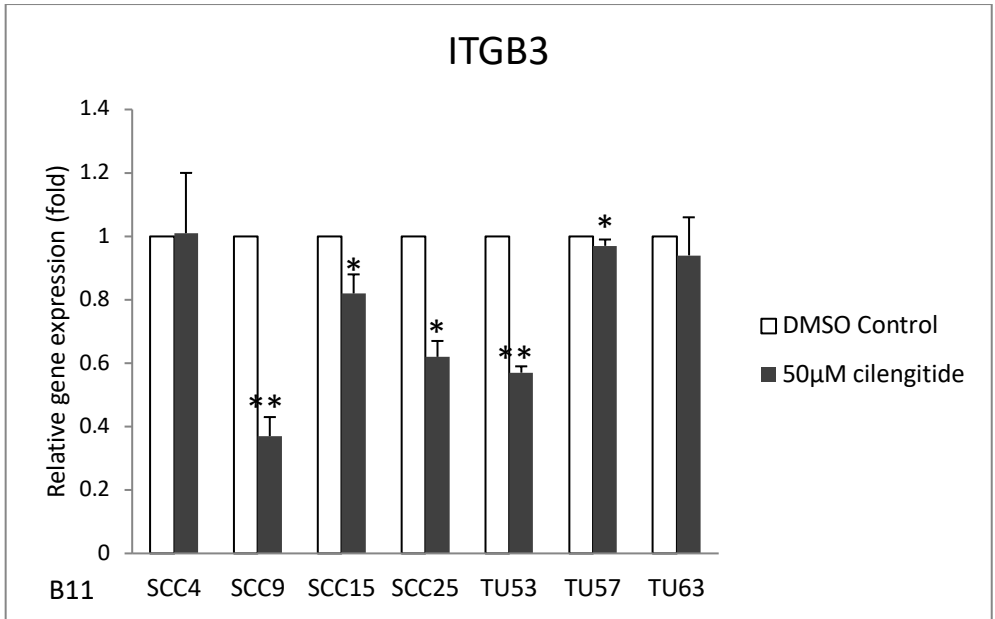
**Figure 10:** Cilengitide or vismodegib suppressed HNSCC cell proliferation. Cells were plated at a density of  $1 \times 10^5$  cells per well in a 96-well plate and were allowed to attach before incubation in medium containing cilengitide (A and B) or vismodegib (C and D) for 72 h. Absorbance at 450 nm was assessed using a BrdU test. Mean  $\pm$  SEM (n=8). \*  $P < 0.05$ , \*\*  $P < 0.01$  (compared to the control). Proliferation of cell lines and primary cell cultures was suppressed by cilengitide (A and B) or vismodegib (C and D) in a dose dependent manner.

### 3.4.3 Real time PCR

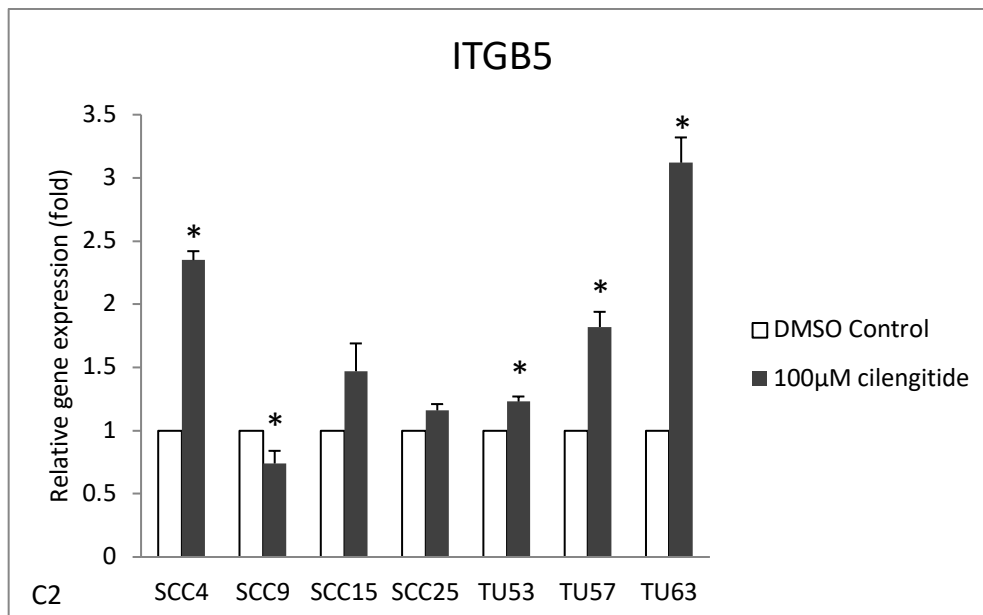
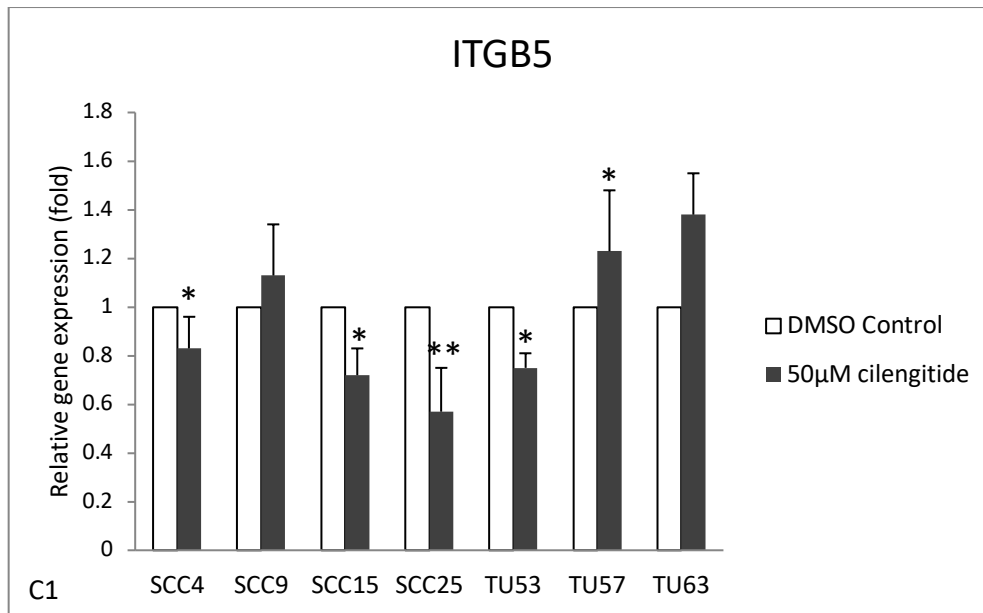
#### 3.4.3.1 Cilengitide

Results showed that 50  $\mu$ M cilengitide led to significantly reduced expression of the integrin target genes ITGAV, ITGB3, and ITGB5 (Fig. 11: A1, A2 and A3) compared with the control. But incubation with 100  $\mu$ M cilengitide led to significantly increased expression of those genes both in cell lines and primary cell cultures (Fig. 11: B1, B2 and B3).





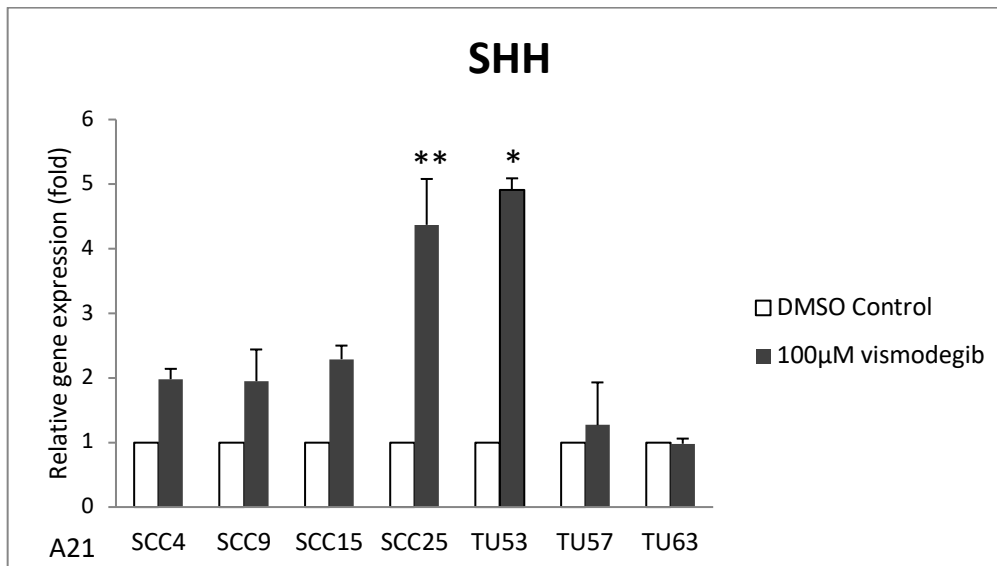
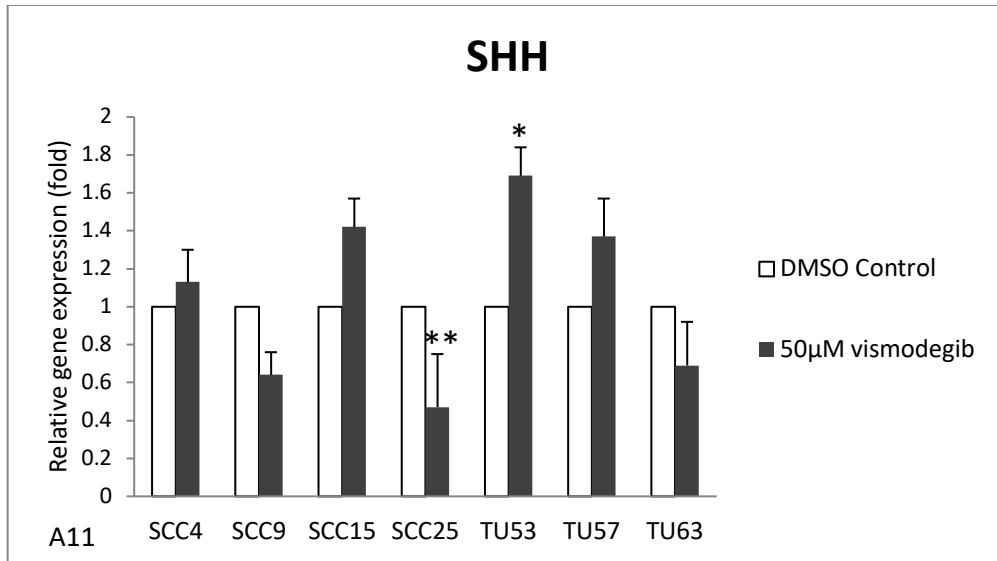


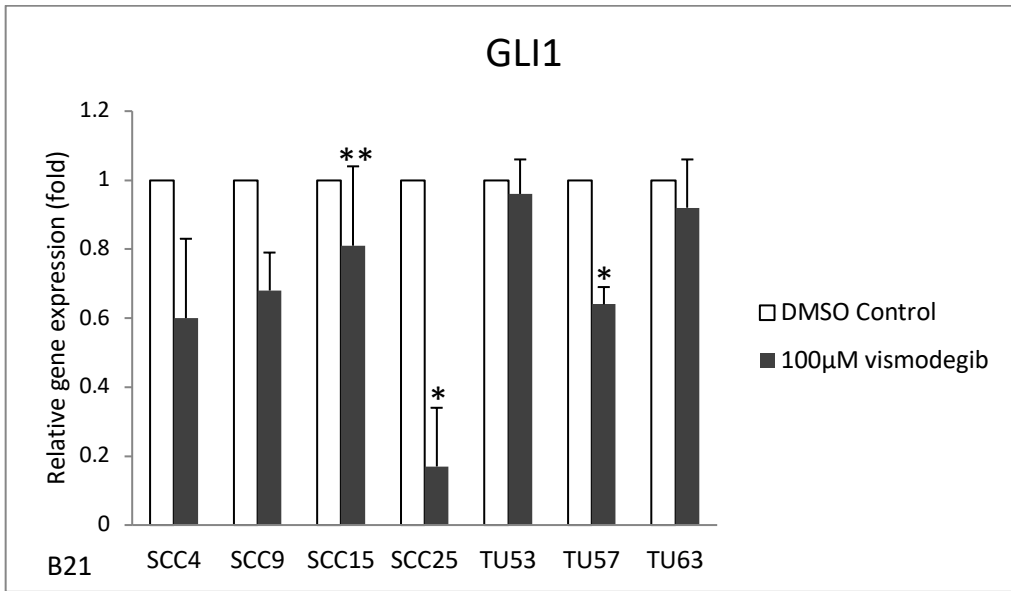
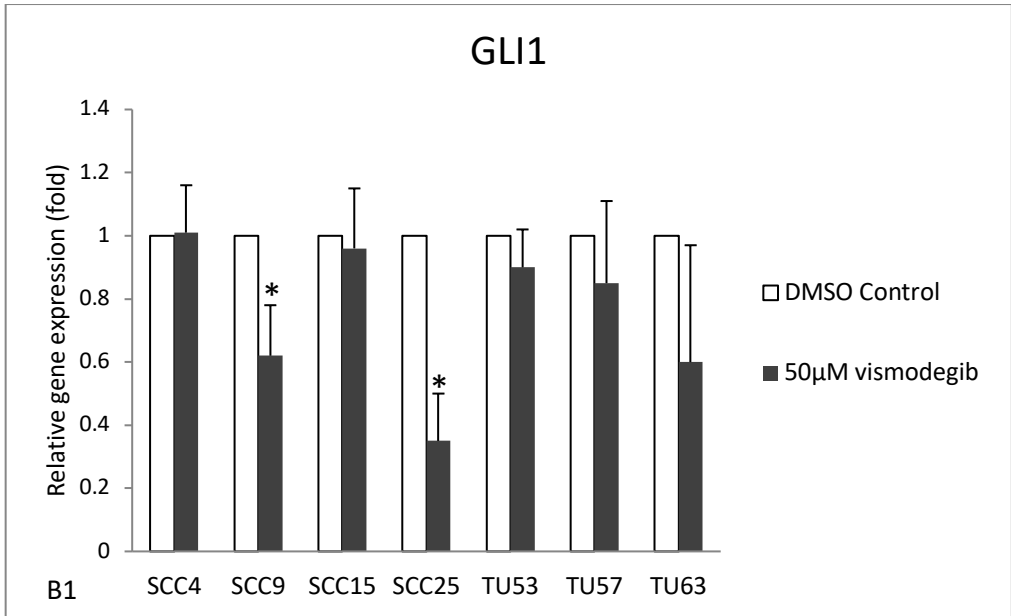


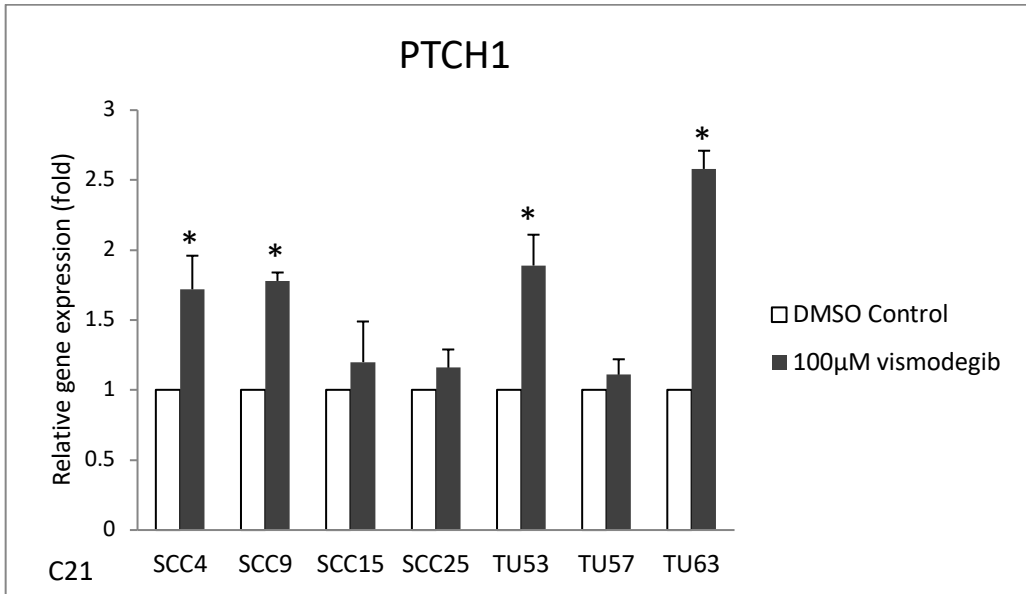
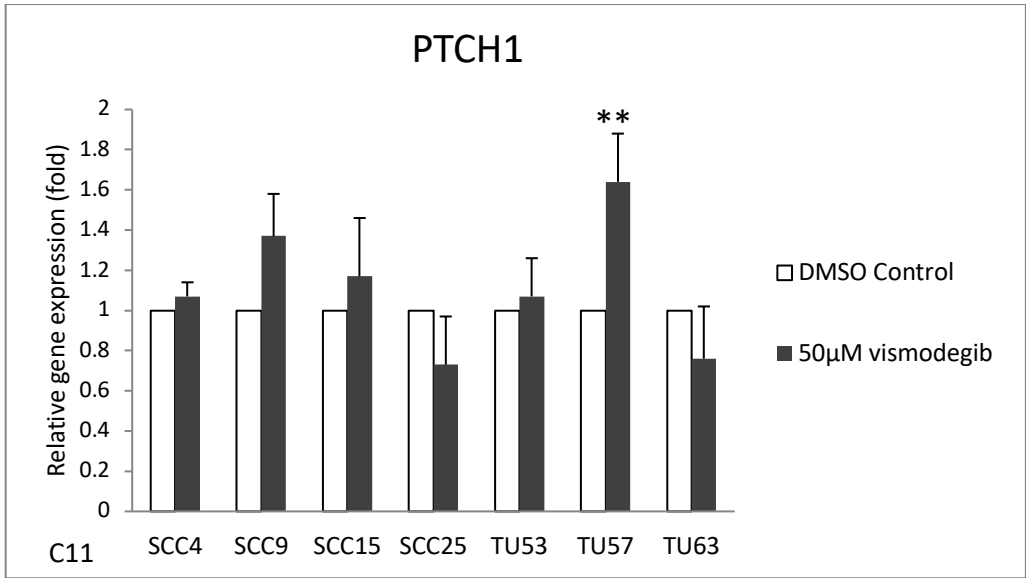
**Figure 11:** Effect of cilengitide on the expression of integrin target genes. *GAPDH*-normalized mRNA levels of ITGAV, ITGB3, ITGB5 revealed by real-time PCR in cell lines and primary cell cultures after 72 h of treatment with cilengitide at a concentration of 50 µM (A1, B1, C1) or 100 µM (A2, B2, C2). Gene expression was normalized to that of endogenous *GAPDH* in each cell line. Data represent the mean ± SD of three independent experiments. (Two-tailed t test, \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ )

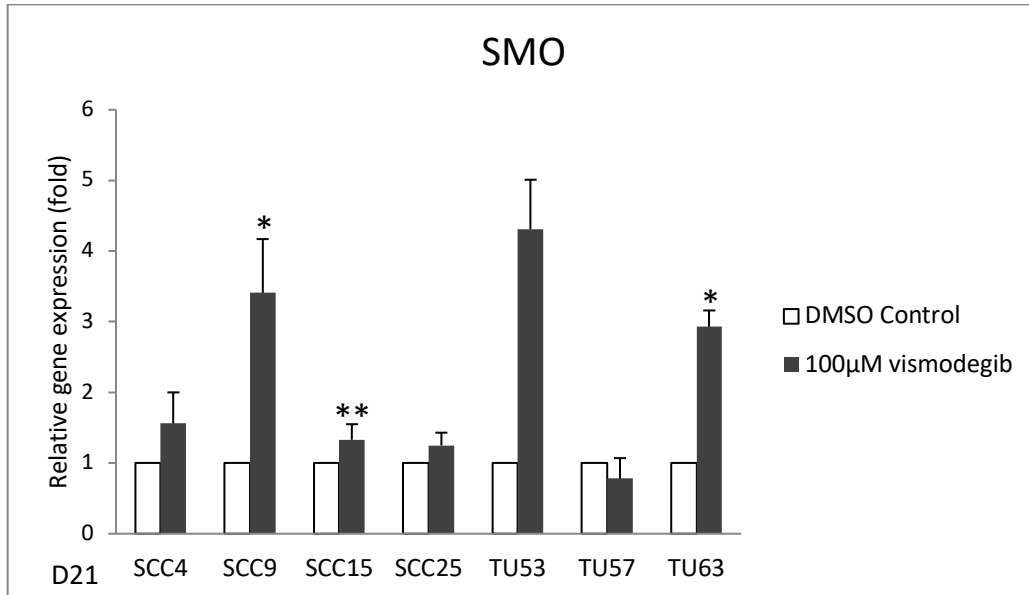
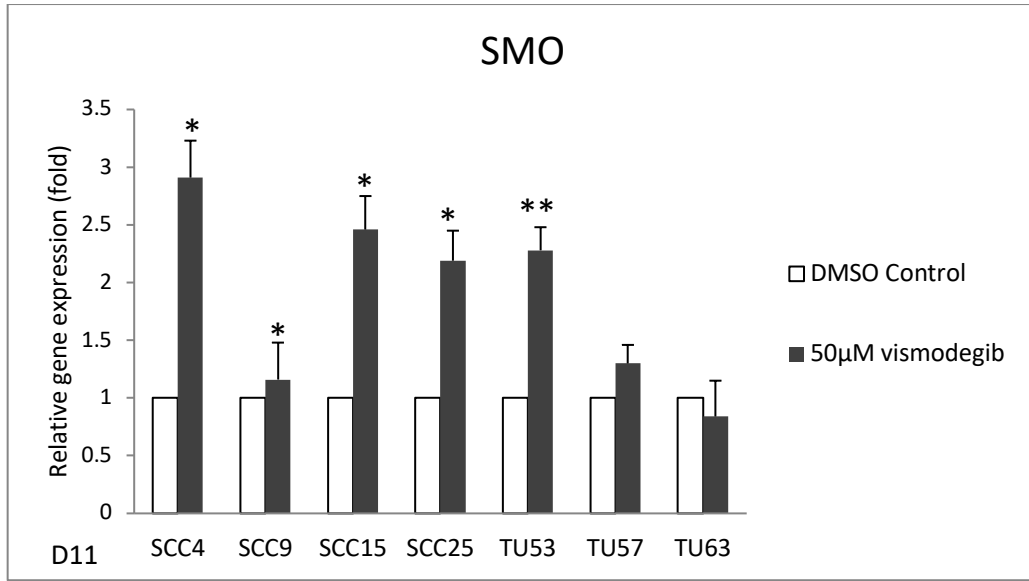
### 3.4.3.2 Vismodegib

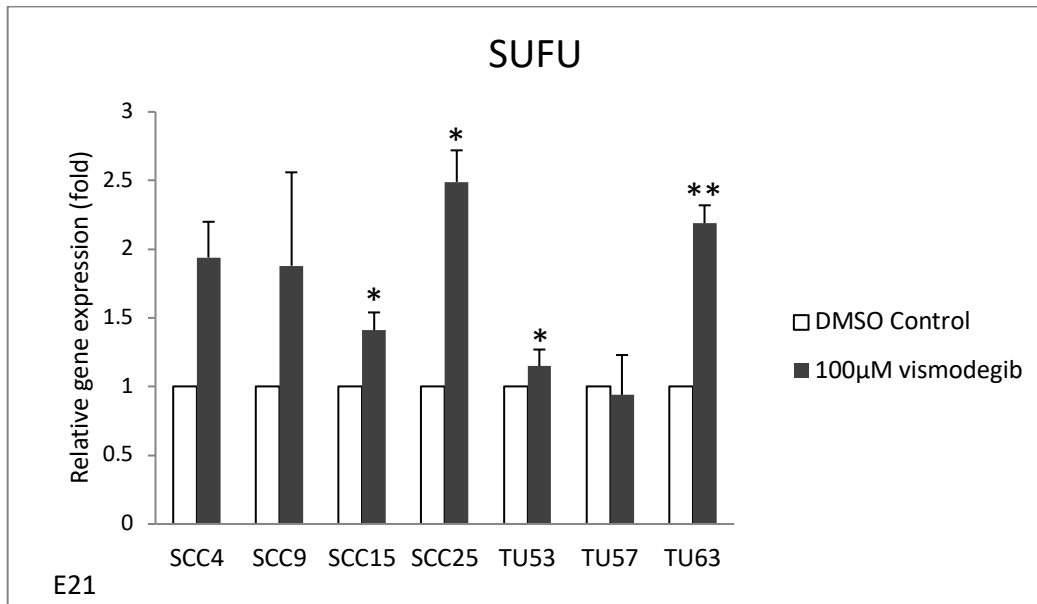
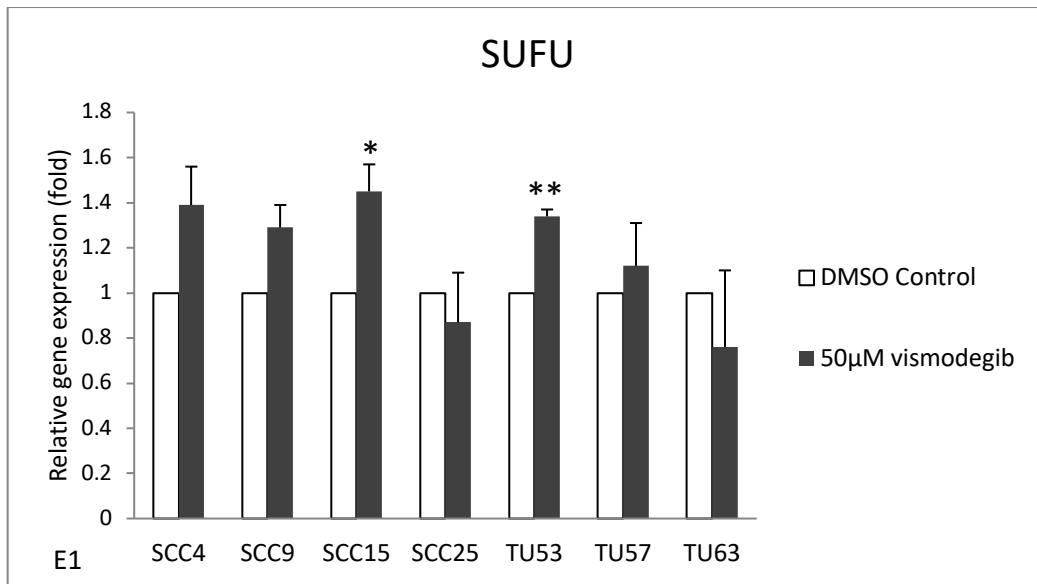
Results showed that 50  $\mu$ M or 100  $\mu$ M vismodegib led to significantly reduced GLI1 gene expression (Fig. 12: B1, and B2), as target gene expression was less than that of the control. However, incubation with 100  $\mu$ M vismodegib led to increased expression of SHH, PTCH1, SMO, and SUFU both in cell lines and primary cell cultures (Fig. 12: A2, C2, D2 and E2).











**Figure 12:** Effect of vismodegib on the expression of integrin target genes. A1, B1, C1, D1, E1, F1: *GAPDH*-normalized mRNA levels of SHH, PTCH1, SMO, SUFU and GLI1 revealed by real-time PCR in cells treated with DMSO or 50 µM vismodegib for 72 h. A2, B2, C2, D2, E2, F2: *GAPDH*-normalized mRNA levels of SHH, PTCH1, SMO, SUFU and GLI1 revealed by real-time PCR in cells treated with DMSO or 100 µM vismodegib for 72 h. Gene expression was normalized to that of endogenous *GAPDH* in each cell line. Data represent the mean ± SD of three independent experiments. (Two-tailed t test, \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ )

## 4. Discussion

### 4.1 Cilengitide

Tumor invasion and metastasis are the main causes of death in cancer patients and are also the key reasons for difficulties in developing radical cures. The tumor metastasis process involves interactions among a variety of cell adhesion molecules, ECM and other blood cells (Liotta and Stetler-Stevenson, 1991). Integrins are important adhesion molecules and play a regulatory role in many critical biological processes such as cell proliferation, invasion, apoptosis, metastasis, and angiogenesis (Varner and Cheresch, 1996, Lester and McCarthy, 1992).

As RGD peptides are non-immunogenic, nontoxic and have no adverse effects, they have emerged as an area of intensive research in the field of inhibition of tumor metastasis through their interaction with integrins. Exogenous RGD peptides can be used as competitive integrin inhibitors to block the downstream signaling pathways of integrins through specific binding to integrins, thereby producing significant inhibition of tumor cell proliferation, adhesion, metastasis and angiogenesis (Jovanovic et al., 2007). Therefore, small peptides containing the RGD sequence have important clinical value in cancer diagnosis and treatment, and have attracted considerable research interest. Researchers have designed and synthesized a series of tripeptide RGD-containing peptides or peptide analogues that inhibit the association of ECM ligand proteins with integrins, for the treatment of malignant tumors (Kumagai et al., 1991). Cilengitide is a synthetic, cyclic peptide containing the RGD sequence (cRGDf (n-Me) V) and is internationally recognized as a small molecule antagonist of integrin  $\alpha\beta3$  and  $\alpha\beta5$  (Ten Hagen et al., 2013). In our present study, we exposed SCC to cilengitide in vitro and studied the effects of cilengitide exposure at different concentrations on cell proliferation. Our results showed that cilengitide could significantly inhibit the proliferation of SCCs in a dose-dependent manner. For a given concentration, cilengitide exerted a stronger suppression effect on the proliferation of primary cell cultures than on cell lines, suggesting that primary cell cultures may retain more of their internal biological characteristics (Manikandan et al., 2016). Thus, the application of drugs to the cell lines or primary cell cultures resulted in different effects.

While cilengitide has entered phase I and phase II clinical trials for some malignant tumors, such as malignant glioma (Nabors et al., 2007, MacDonald et al., 2008, Albert et al., 2006), research about its application to head and neck cancer, especially oral squamous carcinoma, is just beginning, and no data are currently available. Although a small proportion of patients with glioma respond to cilengitide when it is administered at high doses (Nabors et al., 2007), there is currently little evidence that it is effective in the treatment of other human cancers (Hariharan et al., 2007, Gerstner et al., 2015, Vansteenkiste et al., 2015). No study has addressed whether cilengitide-conferred inhibition of the expression of integrin can suppress proliferation and induce apoptosis of oral squamous carcinoma cells.

It remains unknown whether RGD peptides, as competitive integrin inhibitors, can directly inhibit integrin gene expression. In this study, we used RT-PCR to detect mRNA expression of ITGAV, ITGB3 and ITGB5 before and after exposure to cilengitide. Our results showed that 50  $\mu$ M cilengitide led to significantly reduced expression of ITGAV, ITGB3 and ITGB5. But, interestingly, with 100  $\mu$ M cilengitide, expression of ITGAV, ITGB3 and ITGB5 increased significantly both in cell lines and primary cell cultures. One possible explanation for this result is that integrin overexpression is associated with chemotherapy resistance that could prevent patients from achieving complete remission. RGD peptides are competitive inhibitors which impede the binding of integrins to their ECM ligands through their association with integrins, thereby blocking biological events downstream of the integrin-mediated pathways and inhibiting tumor invasion and metastasis. The mechanism by which integrin expression is inhibited following exposure to RGD peptides remains undefined. A possible explanation may be that the binding between RGD peptides and integrin induces changes in the conformation and molecular weight of integrin, resulting in undetectable integrin expression. In addition, there may exist negative and positive feedback mechanisms acting on the expression level of integrins on the cell surface and their ECM ligands. Exogenous RGD peptides might bind to integrins in large quantities, leaving a large number of integrin ligands in the ECM unoccupied, thereby creating negative feedback inhibition on the secretion of integrins, hence reducing integrin expression. Or exogenous RGD peptides might bind to integrins in huge quantities covering all integrins, thereby leaving free a huge number of integrin ligands in the ECM, creating positive feedback on the secretion of integrins and hence



increasing integrin expression. Currently, no conclusive experimental evidence is available to support these possibilities and further research is needed.

Reynolds et al (2009) found evidence in vivo that low (nanomolar) concentrations of cilengitide can paradoxically stimulate tumor growth and angiogenesis by altering  $\alpha v \beta 3$  integrin and vascular endothelial growth factor receptor-2 trafficking, thereby promoting endothelial cell migration to VEGF. Research had already shown that  $\alpha v \beta 5$ , but not  $\alpha v \beta 3$ , is overexpressed in HNSCC tissue, whereas both integrins are expressed on sprouting vessels (Fabricius et al., 2011). Preclinical studies in mice bearing xenografts (A431 epidermoid carcinoma or U87MG glioblastoma cells) demonstrated a synergistic effect between cetuximab (10 mg/kg intra-peritoneal weekly) and cilengitide (25 mg/kg intra-peritoneal 5 $\times$ /week). The phase I part of the advantage study demonstrated cilengitide plus cisplatin, 5-FU, and cetuximab (PFE) was well tolerated by patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck (Vermorken et al., 2011). The most common cilengitide-related adverse events included nausea, anorexia, and asthenia, but no dose-limiting or unexpected toxicities were reported. Phase I/II trials showed the potential of cilengitide activity as a single-agent therapy in recurrent glioblastoma multiforme (Reardon et al., 2008), and as a concomitant and adjuvant therapy with standard chemoradiotherapy in newly diagnosed glioblastoma multiforme (Stupp et al., 2010, Nabors et al., 2012). Furthermore, in phase II of a randomized phase I/II advantage trial, Vermorken et al. (2014) showed that, compared with cetuximab and platinum-based chemotherapy alone, the addition of cilengitide with cisplatin, 5-fluorouracil, and cetuximab was well tolerated, but did not result in a better outcome. Therefore, this combination cannot be recommended for further development in treating patients with recurrent/metastatic squamous cell carcinoma of the head and neck. Controversially, some research showed the results of combination therapy, either with irradiation or other drug chemotherapy (Heiduschka et al., 2014, Eisele et al., 2014, Vansteenkiste et al., 2015), had better outcomes than monotherapy. In summary, RGD peptides can inhibit tumor cell proliferation and hold tremendous promise in tumor chemotherapy. However, research on the role of RGD peptides in cancer therapy is still in its early stages and the underlying mechanism is still poorly understood (Vansteenkiste et al., 2015). The key issue is how to identify more integrin ligands with higher affinity, namely peptides containing the RGD sequence. As phage library techniques become increasingly sophisticated and novel screening techniques

continue to be adopted, we believe that RGD-containing small molecule synthetic peptides will be seriously considered in tumor chemotherapy in the near future and look set to offer broader chemotherapy drug options for HNSCC.

## 4.2 Vismodegib

Overexpressed or mutated proteins in the HH pathway or activation of the related transcription factors are apparent in a variety of human malignancies, including HNSCC, and are associated with poor prognosis (Cavicchioli Buim et al., 2011, Yan et al., 2011, Thomas et al., 2005), so this study is of particular importance (Onishi and Katano, 2011). Inappropriate activation of HH signaling by overexpression or mutation was found to increase snail protein expression, with a loss of cellular adhesion, activate anti-apoptotic genes and angiogenic factors, reduce apoptotic gene activation, and interfere in the cell-cycle sequence (Galimberti et al., 2012, Kim et al., 2014). These HH-mediated properties may contribute to tumor development and facilitate tumor growth and spread, with consequent poor prognosis for different human malignancies. In healthy adult tissues, the HH pathway regulates stem cells during regeneration and tissue repair (Beachy et al., 2004, Mimeault and Batra, 2006), but disturbances within this system may easily lead to cancer cell formation. Tumor repopulation after chemotherapy might be promoted by HH signaling, with the consequences of early recurrence, treatment resistance, and poor prognosis (Chen et al., 2007, Liu et al., 2014, Varnat et al., 2009). Jaggupilli and Elkord (2012) reported HH pathway overexpression (SMO and GLI1) in putative HNSCC cancer stem cell subpopulations (CD24, CD44 and CD133), although there is currently no agreed consensus about stem cell markers in HNSCC. Data from studies of HH protein expression in HNSCC have been reported, but the samples sizes were small, not all components were considered (Schneider et al., 2011), and results concerning expression patterns were partly controversial (Wang et al., 2012). A recent study demonstrated increased expression of most HH pathway proteins, especially GLI1. Vismodegib (GDC-0449) is a synthetic HH inhibitor. The substance acts as a cyclopamine-competitive antagonist of the smoothed receptor (SMO) which is part of the HH signaling pathway. SMO inhibition causes the transcription factors GLI1 and GLI2 to remain inactive, which prevents the expression of tumor mediating genes within the pathway. Phase II trials with vismodegib have been successfully performed

for skin, brain, and breast tumors (see the listing of current clinical trials of the National Cancer Institute, [www.cancer.gov](http://www.cancer.gov)), and other tumor entities are currently under investigation. In a phase II, randomized, double-blind, placebo-controlled trial, vismodegib was tested to provide an estimate of efficacy in the setting of second or third complete remission in 104 ovarian cancer patients (Kaye et al., 2012). Although numerical improvements were observed, a clinically meaningful improvement in progression-free survival for vismodegib versus placebo maintenance could not be demonstrated, and HH ligand-expression frequency was lower than expected. One possible explanation for this result is that HH ligand overexpression is associated with chemotherapy resistance that could prevent patients from achieving complete remission. Alternatively, it is conceivable that HH ligand overexpression is actually associated with a lower probability of relapse, leading to a lower prevalence of HH-positive ovarian cancer in the patients of this study (Kaye et al., 2012). This report stressed the importance of an appropriate patient selection strategy for further studies. In another analysis of a multi-center, randomized, double-blind, placebo-controlled, phase II trial in patients with the basal-cell nevus (Gorlin) syndrome, results indicated that vismodegib reduces basal-cell carcinoma tumor burden and prevents new basal-cell carcinoma growth in patients with basal-cell nevus syndrome. Adverse events associated with vismodegib frequently led to interruption of treatment, followed by basal-cell carcinoma recurrence (Tang et al., 2016). Until now, no clinical trials of vismodegib have been carried out on patients with HNSCC.

As reported in our study, SMO inhibition by vismodegib has demonstrated a potential inhibitory effect against tumor growth in tumor cell lines and primary cell cultures. The inhibitory effect was more pronounced on primary cell cultures than on cell lines. Vismodegib treatment also led to down-regulation of the HH target gene *GLI1*. But our study has demonstrated that 100  $\mu$ M vismodegib resulted in up-regulation of *SHH*, *SMO*, *PTCH* and *SUFU*. One possible explanation for this result is that HH ligand overexpression is associated with chemotherapy resistance that could prevent patients from achieving complete remission. Bahra *et al.* reported on the synergistic effects of cyclophosphamide and gemcitabine in the reduction of tumor volume in pancreatic adenocarcinoma xenografts and postulated a new therapeutic approach (Bahra et al., 2012). Lapatinib suppresses colony formation of epithelial HNSCC cells, and in combination with cisplatin, its efficacy is increased (Schrader et al., 2012). Liebig *et al.* (2017) provided ex-vivo evidence for the potential of vismodegib

in HNSCC therapies, especially if combined with cetuximab, cisplatin and docetaxel. Vismodegib suppressed proliferation and colony formation. This means that combined treatment with the guideline cytostatic drugs cisplatin or docetaxel synergistically contributed to even greater inhibition of colony formation of HNSCC than single drug therapy.

In conclusion, the direct blockade of an activated pathway that is responsible for, or at least involved in, tumor growth is a promising goal in cancer therapy. Also, combinations of drugs targeting SMO with guideline-conforming cytostatic drugs may be superior to single agent therapy. According to our results, assuming vismodegib can be introduced into clinical practice, it is quite possible that a promising benefit in HNSCC could also be expected. Our results encourage further investigation concerning the HH pathway in HNSCC and the development of new agents targeting this signaling cascade with less serious adverse effects than vismodegib.

## 5. Conclusion

Due to the low 5-year survival rate and the high recurrence rate of HNSCC, new chemotherapeutic strategies are of high clinical interest. Integrins are important adhesion molecules and play a regulatory role in many critical biological processes such as cell proliferation, invasion, apoptosis, metastasis, and angiogenesis in squamous cell carcinoma. Cilengitide is a cyclized RGD containing pentapeptide that selectively blocks activation of the integrins. Recently, growing evidence has suggested aberrant activation of the HH pathway in squamous cell carcinoma plays a critical role during cancer development. Vismodegib (GDC-0449) is an orally administered agent that selectively suppresses SMO activity.

Most previous studies used cell lines purchased from commercial companies. These cell lines may already have differentiated and lost some of their potential characteristics as tumor cells. Accordingly, we isolated and identified primary HNSCC cell cultures, and evaluated the effects of cilengitide and vismodegib on both cell lines and primary cell cultures. The application of drugs to the cell lines or primary cell cultures produced different results.

Four tongue squamous cell carcinoma cell lines (SCC4, SCC9, SCC15 and SCC25) were obtained from the American Type Culture Collection. Three primary cell cultures (TU53, TU57 and TU63) were obtained, respectively, from three patients with HNSCC, who underwent surgery. DNA profiling using STRs showed that K53 and TU53, K57 and TU57, K63 and TU 63 are all homologous.

To identify the biological characteristics of the primary cell cultures and cell lines, four kinds of typical immunocytochemical staining were performed. All cells expressed negative CD90, excluding a fibroblast source. Both cell lines and primary cell cultures strongly expressed cytokeratin, which is characteristic of epithelial cells. All cells showed moderate expression of vimentin, demonstrating a mesenchymally derived sarcoma tumor. All cells expressed SHH, indicating activation of the HH pathway in HNSCC.

MTT and BrdU tests showed proliferation of cell lines and primary cell cultures were suppressed by cilengitide or vismodegib in a dose dependent manner without exception. Primary cell cultures expressed more significantly depressed growth than cell lines. 6.25  $\mu\text{M}$  of cilengitide had a significant inhibitory effect on primary cell

cultures, but a concentration as high as 100  $\mu\text{M}$  was needed to produce a similar effect on cell lines. 50  $\mu\text{M}$  vismodegib significantly inhibited the proliferation of primary cell cultures, but for cell lines the concentration needed to be as high as 100  $\mu\text{M}$ . Primary cell cultures may retain more of their internal biological characteristics. Therefore, individualized treatment may be necessary with regard to the different stages of tumor progression.

RT-PCR results showed that 50  $\mu\text{M}$  cilengitide significantly reduced ITGAV, ITGB3, and ITGB5 gene expression. But incubation with 100  $\mu\text{M}$  cilengitide significantly increased expression of ITGAV, ITGB3, and ITGB5 in all cells. Cilengitide can inhibit tumor cell proliferation and integrin gene expression at low concentrations, holding tremendous promise in tumor chemotherapy. However, the research on the role of RGD peptides in cancer therapy is still in its early stages and further investigations are required.

RT-PCR results also showed that GLI1 gene expression was reduced significantly by incubation with 50  $\mu\text{M}$  or 100  $\mu\text{M}$  vismodegib. The results demonstrated that vismodegib could effectively block activation of HH signal pathway.

Findings from current study have potentially important clinical implications. We found that the integrin inhibitor cilengitide and HH signal inhibitor vismodegib can inhibit HNSCC cell proliferation and reduce target gene expression at certain concentrations. Patients with HNSCC tumors may benefit from an individualized therapeutic strategy.

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# 7. Appendix

**Table 12:** *MTT tests on different cells with different concentration of cilengitide (72 h) (Means ± SD)*

Cilengitide (µM)	0	6.25	12.5	25	50	100
SCC4	1.014±0.069	1.007±0.081	1.024±0.125	1.059±0.066	0.938±0.073	0.755±0.027
SCC9	1.009±0.058	1.020±0.061	1.006±0.051	0.937±0.038	0.802±0.038	0.641±0.082
SCC15	1.004± 0.114	0.876± 0.076	0.901±0.069	0.884± 0.041	0.886±0.066	0.452±0.079
SCC25	1.003± 0.045	1.018± 0.039	1.017±0.077	1.027± 0.038	1.016±0.094	0.570± 0.070
TU53	1.003± 0.057	0.969±0.040	0.961± 0.052	0.967± 0.037	0.879±0.045	0.453±0.032
TU57	1.003±0.068	0.590± 0.032	0.542±0.046	0.554±0.076	0.535± 0.056	0.433±0.017
TU63	0.994± 0.054	0.672± 0.071	0.484±0.050	0.409±0.027	0.362± 0.017	0.364± 0.040

**Table 13:** *MTT tests on different cells with different concentration of vismodegib (72 h) (Means ± SD)*

Vismodegib (µM)	0	6.25	12.5	25	50	100
SCC4	1.006±0.035	0.985±0.070	0.955±0.071	0.959±0.052	0.894±0.097	0.760±0.021
SCC9	1.003±0.060	0.954±0.056	0.924±0.093	0.923±0.063	0.860±0.058	0.756±0.059
SCC15	1.004±0.114	0.928±0.076	0.885±0.101	0.943±0.058	0.908±0.057	0.536±0.044
SCC25	1.003± 0.045	0.972±0.053	1.022±0.038	0.934±0.038	0.894±0.063	0.365±0.038
TU53	1.002±0.039	1.001±0.035	0.977±0.035	0.950±0.046	0.884±0.047	0.466±0.055
TU57	0.998±0.060	0.917±0.032	0.928±0.043	0.883±0.060	0.802±0.022	0.622±0.024
TU63	0.995±0.062	0.932±0.041	0.906±0.041	0.899±0.056	0.881±0.055	0.775±0.049

**Table 14:** *BrdU tests on different cells with different concentration of cilengitide (72 h) (Means ± SD)*

Cilengitide (μM)	0	6.25	12.5	25	50	100
SCC4	1.000±0.056	1.075±0.027	1.044±0.044	1.045±0.038	1.040±0.053	0.807±0.034
SCC9	1.000±0.079	1.031±0.041	0.950±0.052	0.967±0.057	0.895±0.076	0.887±0.100
SCC15	1.000±0.069	1.030±0.023	1.022±0.044	1.027±0.061	1.008±0.036	0.587±0.100
SCC25	1.000±0.042	0.922±0.048	1.011±0.043	1.031±0.052	1.078±0.034	0.643±0.082
TU53	1.000±0.084	0.970±0.048	1.021±0.050	0.969±0.064	1.014±0.043	0.696±0.061
TU57	1.000±0.091	0.797±0.128	0.690±0.138	0.633±0.094	0.544±0.090	0.336±0.096
TU63	1.000±0.114	0.887±0.096	0.670±0.075	0.407±0.032	0.374±0.019	0.328±0.013

**Table 15:** *BrdU tests on different cells with different concentration of vismodegib (72 h) (Means ± SD)*

Vismodegib (μM)	0	6.25	12.5	25	50	100
SCC4	1.000±0.032	1.034±0.036	0.949±0.139	0.991±0.041	0.945±0.051	0.789±0.041
SCC9	1.000±0.073	0.956±0.092	0.918±0.081	0.892±0.083	0.843±0.044	0.765±0.049
SCC15	1.000±0.035	0.934±0.047	0.913±0.072	0.854±0.058	0.812±0.028	0.533±0.028
SCC25	1.000±0.073	0.971±0.047	0.960±0.039	0.948±0.048	0.843±0.039	0.487±0.228
TU53	1.000±0.078	1.010±0.067	0.956±0.070	0.964±0.058	0.907±0.069	0.695±0.066
TU57	1.000±0.057	0.976±0.058	0.917±0.097	0.884±0.065	0.781±0.059	0.524±0.032
TU63	1.000±0.114	0.966±0.127	0.958±0.237	1.004±0.084	0.694±0.161	0.611±0.144



**Table 16:** *Relative mRNA expression level of genes of cells with 50  $\mu$ M cilengitide detected by qPCR (72 h)*

	SCC4	SCC9	SCC15	SCC25	TU53	TU57	TU63
ITGAV	1.17 $\pm$ 0.06	1.00 $\pm$ 0.09	0.87 $\pm$ 0.26	0.70 $\pm$ 0.03	0.92 $\pm$ 0.04	1.08 $\pm$ 0.15	0.86 $\pm$ 0.32
ITGB3	1.01 $\pm$ 0.19	0.37 $\pm$ 0.06	0.82 $\pm$ 0.06	0.62 $\pm$ 0.05	0.57 $\pm$ 0.02	0.97 $\pm$ 0.02	0.94 $\pm$ 0.12
ITGB5	0.83 $\pm$ 0.13	1.13 $\pm$ 0.21	0.72 $\pm$ 0.11	0.57 $\pm$ 0.18	0.75 $\pm$ 0.06	1.23 $\pm$ 0.25	1.38 $\pm$ 0.17

**Table 17:** *Relative mRNA expression level of genes of cells with 100  $\mu$ M cilengitide detected by qPCR (72 h)*

	SCC4	SCC9	SCC15	SCC25	TU53	TU57	TU63
ITGAV	4.38 $\pm$ 0.13	1.05 $\pm$ 0.04	1.98 $\pm$ 0.38	1.72 $\pm$ 0.18	1.48 $\pm$ 0.18	1.55 $\pm$ 0.25	1.44 $\pm$ 0.05
ITGB3	8.30 $\pm$ 1.06	0.79 $\pm$ 0.20	3.51 $\pm$ 0.27	7.15 $\pm$ 0.26	3.04 $\pm$ 0.62	0.71 $\pm$ 0.14	8.48 $\pm$ 0.61
ITGB5	2.35 $\pm$ 0.07	0.74 $\pm$ 0.10	1.47 $\pm$ 0.22	1.16 $\pm$ 0.05	1.23 $\pm$ 0.04	1.82 $\pm$ 0.12	3.12 $\pm$ 0.20

**Table 18:** *Relative mRNA expression level of genes of cells with 50  $\mu$ M vismodegib detected by qPCR (72 h)*

	SCC4	SCC9	SCC15	SCC25	TU53	TU57	TU63
SHH	1.13 $\pm$ 0.17	0.64 $\pm$ 0.12	1.42 $\pm$ 0.15	0.47 $\pm$ 0.28	1.69 $\pm$ 0.15	1.37 $\pm$ 0.20	0.69 $\pm$ 0.23
PTCH1	1.07 $\pm$ 0.07	1.37 $\pm$ 0.21	1.17 $\pm$ 0.29	0.73 $\pm$ 0.24	1.07 $\pm$ 0.19	1.64 $\pm$ 0.24	0.76 $\pm$ 0.26
SMO	2.91 $\pm$ 0.32	1.16 $\pm$ 0.32	2.46 $\pm$ 0.29	2.19 $\pm$ 0.26	2.28 $\pm$ 0.20	1.30 $\pm$ 0.16	0.84 $\pm$ 0.31
SUFU	1.39 $\pm$ 0.17	1.29 $\pm$ 0.10	1.45 $\pm$ 0.12	0.87 $\pm$ 0.22	1.34 $\pm$ 0.03	1.12 $\pm$ 0.19	0.76 $\pm$ 0.34
GLII	1.01 $\pm$ 0.15	0.62 $\pm$ 0.16	0.96 $\pm$ 0.19	0.35 $\pm$ 0.15	0.90 $\pm$ 0.12	0.85 $\pm$ 0.26	0.60 $\pm$ 0.37

**Table 19:** *Relative mRNA expression level of genes of cells with 100  $\mu$ M vismodegib detected by qPCR (72 h)*

	SCC4	SCC9	SCC15	SCC25	TU53	TU57	TU63
SHH	1.98 $\pm$ 0.16	1.95 $\pm$ 0.49	2.29 $\pm$ 0.21	4.37 $\pm$ 0.71	4.91 $\pm$ 0.18	1.27 $\pm$ 0.66	0.98 $\pm$ 0.08
PTCH1	1.72 $\pm$ 0.24	1.78 $\pm$ 0.06	1.20 $\pm$ 0.29	1.16 $\pm$ 0.13	1.89 $\pm$ 0.22	1.11 $\pm$ 0.11	2.58 $\pm$ 0.13
SMO	1.56 $\pm$ 0.44	3.41 $\pm$ 0.76	1.33 $\pm$ 0.22	1.25 $\pm$ 0.18	4.31 $\pm$ 0.7	0.78 $\pm$ 0.29	2.93 $\pm$ 0.23
SUFU	1.94 $\pm$ 0.26	1.88 $\pm$ 0.68	1.41 $\pm$ 0.13	2.49 $\pm$ 0.23	1.15 $\pm$ 0.12	0.94 $\pm$ 0.29	2.19 $\pm$ 0.13
GLII	0.60 $\pm$ 0.23	0.68 $\pm$ 0.11	0.81 $\pm$ 0.23	0.17 $\pm$ 0.17	0.96 $\pm$ 0.1	0.64 $\pm$ 0.05	0.92 $\pm$ 0.14

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## 9. Publication

A comparative assessment of the effects of integrin inhibitor cilengitide on primary culture of head and neck squamous cell carcinoma (HNSCC) and HNSCC cell lines.

**L. Zhang** · A. Gülses · N. Purcz · J. Weimer · J. Wiltfang · Y. Açil.

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### **A comparative assessment of the effects of integrin inhibitor cilengitide on primary culture of head and neck squamous cell carcinoma (HNSCC) and HNSCC cell lines.**

Zhang L<sup>1</sup>, Gülses A<sup>2</sup>, Purcz N<sup>3</sup>, Weimer J<sup>4</sup>, Wiltfang J<sup>3</sup>, Açil Y<sup>3</sup>.

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#### **Abstract**

**BACKGROUND:** Integrins are highly attractive targets in oncology due to their involvement in angiogenesis in a wide spectrum of cancer entities. Among several integrin inhibitors, cilengitide is suggested to be one of the most promising inhibitors. However, little is known about the cellular processes induced during cilengitide chemotherapy in head and neck squamous cell carcinoma (HNSCC).

**MATERIALS AND METHODS:** For the current study, 3 HNSCC cell lines, SCC4, SCC15 and SCC25; and 3 primary culture cells, TU53, TU57, and TU63 were used. CD90, cytokeratin, and vimentin were stained immunohistochemically to identify the biological characteristics of these cell lines and primary culture cells and the cytostatic effect of cilengitide was evaluated. Quantitative polymerase chain reaction (qPCR) arrays were applied to evaluate target protein genes ITGAV, ITGB3, and ITGB5 of integrin  $\alpha v\beta 3$  and  $\alpha v\beta 5$  at respective concentrations of 50 and 100  $\mu\text{M}$  cilengitide for 72 h.

**RESULTS:** Cilengitide has significantly inhibited the proliferation of HNSCC cells in a dose-dependent way. At the same concentration, cilengitide suppressed the proliferation of primary culture cells even more strongly than it did that of cell lines, suggesting that primary culture cells retain more of their internal biological characteristics than do cell lines. qPCR assay detected downregulation of ITGAV, ITGB3, and ITGB5 gene expression after exposure to 50  $\mu\text{M}$  of cilengitide. However, after exposure to 100- $\mu\text{M}$  cilengitide, expression of these genes significantly increased both in cell lines and primary culture cells.

**CONCLUSIONS:** RGD-containing small-molecule synthetic peptides might be considered in tumor chemotherapy in the near future. The different reactions of primary culture cells and cell lines demonstrated that individualized chemotherapy plans may be a feasible option. However, research on the role of cilengitide in HNSCC therapy is still in its early stages, and further investigations are required.

**KEYWORDS:** Cell line; Cilengitide; Integrin; Primary culture cell; Proliferation; Squamous cell carcinoma

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