Targeting mTOR and AREG with Everolimus, Sunitinib and Sorafenib in HPV-positive and -negative SCC

CHRISTOPH ADERHOLD, ANNE FABER, CLAUDIA UMBREIT, RICHARD BIRK, CHRISTEL WEISS, JÖRG ULRICH SOMMER, KARL HÖRMANN and JOHANNES DAVID SCHULTZ

Department of Otorhinolaryngology Head and Neck Surgery, University Hospital Mannheim, Mannheim, Germany

Abstract. *Background/Aim: Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy. It is the most common neoplasm appearing in the upper aerodigestive tract and the sixth most common cancer worldwide. The five-year survival rate remains poor despite advances in surgery, radiation and chemotherapy. Furthermore, the incidence of human papillomavirus (HPV)-associated oropharyngeal cancer is rising. Thus, innovative therapy approaches are imperative in order to improve the situation. Everolimus, an inhibitor of the mammalian target of rapamycin (mTOR) and sorafenib and sunitinib, multityrosine kinase inhibitors, have been notably effective in the therapy of different tumor entities. The modest side-effects and oral application of the drugs might improve patient compliance. Expression levels of mTOR and Amphiregulin (AREG) in p16-positive and -negative SCC (squamous cell carcinoma) and the effect of everolimus, sorafenib or sunitinib on the expression levels of these target proteins were assessed. As far as we are aware of, this is one of the first in vitro studies to evaluate the effect of these small-molecule drugs with regard to the p16 status of SCC cells. Materials and Methods: p16-negative HNSCC 11A and 14C cells and p16-positive CERV196 cells were exposed to different concentrations of everolimus, sorafenib and sunitinib for 2-8 days. Expression levels of mTOR and AREG were determined by enzyme-linked immunosorbent assay (ELISA) and compared against a chemonaïve control. Results: AREG and mTOR were expressed in all tested cell lines. CERV196 displayed a remarkable increase of mTOR expression compared to p16-negative HNSCC. On the contrary, AREG levels were reduced by 50% in CERV196. Everolimus, sorafenib and sunitinib significantly reduced mTOR expression. Everolimus significantly decreased AREG expression independently of the HPV status. Sunitinib and sorafenib increased AREG expression in HNSCC 11A and 14C but not in CERV196. Conclusion: The applied drugs showed remarkable suppression of mTOR expression, which might delay tumor progression. Interestingly, sorafenib and sunitinib increased AREG in HNSCC 11A and 14C, which could be a possible evasive mechanism following incubation with these drugs. On the contrary, p16-positive CERV196 showed increased susceptibility to sorafenib and sunitinib concerning suppression of AREG expression. Further studies are required to evaluate the HPV-dependent differences of therapy response and the possible consequences for treatment options.*

Correspondence to: Dr. med. Christoph Aderhold, Department of Otorhinolaryngology Head and Neck Surgery, University Hospital Mannheim, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany. Tel: +49 6213831600, Fax: +49 6213833827, e-mail: christoph.aderhold@umm.de

Key Words: Tyrosin-kinase-inhibitor, mTOR, AREG, head and neck squamous cell carcinoma, everolimus, sorafenib, sunitinib.

Head and neck squamous cell carcinoma (HNSCC) is the most common epithelial malignancy in the upper aerodigetive tract. Every year, this tumor entity accounts for about 686,000 new cases and approximately 375,000 deaths. It is the sixth most common cancer in the world, characterized by variable tumor aggressiveness and response to treatment (1). The overall 5-year survival rate is less than 50% and has not improved despite innovative diagnostics, surgical procedures, radiation and chemotherapy (2). The most important risk factors for HNSCC are smoking and alcohol abuse (3, 4). The recent decrease in HNSCC incidence could be explained by the falling prevalence of tobacco abuse (5).

In contrast, the incidence of oropharyngeal cancer has increased in patients aged 20-44 (6). These oropharyngeal carcinomas are associated with human papillomavirus (HPV) and their rising incidence underlines the importance of the viral etiology (7-9). A systematic review reported an overall HPV-prevalence of about 26% (10). HPV is significantly more common in oropharyngeal HNSCC (35.6%) compared to oral (23.5%) or laryngeal locations (24%) (11). It is remarkable that approximately 95% of HPV-positive HNSCC contain DNA (deoxyribonucleic acid) of the high-risk

subtype 16 (12). The circular, double-stranded DNA genome of HPV encodes three oncoproteins and two structural capsid proteins (13). The oncoprotein E6 degrades tumor suppressor protein p53 (14). At present, over 100 subtypes are known and can be classified as low- or high-risk sub-populations based on their cancerogenic potency (15).

Amphiregulin (AREG) is a ligand of epidermal growth factor receptor (EGFR) and is related to epidermal growth factor (EGF) and transforming growth factor α (TGF-α). Activation of EGFR affects several cellular signal cascades. EGFR can induce tumor angiogenesis and inhibit apoptosis *via* the PI3K (phosphatidylinositol 3-kinase)/Akt signaling pathway (16). Cell cycle progression can be realized *via* activation of the Ras/Raf cascade (17). Over-expression of EGFR can be found in 42-80% of HNSCC and EGFR gene amplification occurs in up to 30% (18-21). This is associated with poorer prognosis (22). The relevance of EGFR and its ligands is illustrated by the fact that an EGFR-targeted therapy with cetuximab is part of the guideline therapy for recurrent or advanced-stage HNSCC with distant metastasis (23). Currently, cetuximab is the only Food and Drug Administration (FDA)-approved targeted-therapy for HNSCC. Mutations of EGFR cause resistance to cetuximab and could be a possible evasive mechanism of the tumor cell during a targeted therapy (24).

The mammalian target of rapamycin (mTOR) is a phosphatidylinositol 3-kinase-related kinase. It integrates the upstream signaling from several messengers including EGFR (25). mTOR detects nutrient, oxygen and energy levels in the cell, as well as oxidative stress. It is part of two functional protein complexes: mTORC1 and mTORC2. mTOR and the PI3K/Akt signaling cascade are closely related. mTORC1, for instance, can be activated *via* the PI3K/Akt signaling pathway. mTORC2, on the other hand, is able to activate Akt (26-28). mTOR alters DNA transcription and protein synthesis of vascular endothelial growth factor (VEGF), and therefore induces angiogenesis (29, 30). Additional functions of mTOR are regulation of proliferation, cell motility, cell survival and protein synthesis.

Given the poor prognosis of HNSCC, new pharmacological approaches are necessary. Tumor heterogeneity gives hope for a personalized anticancer therapy but the only FDA-approved targeted therapy for HNSCC is cetuximab. Personalized therapy approaches are still missing for HNSCC.

The mTOR inhibitor everolimus is used as an immunosuppressant to prevent rejection after organ transplantation (31). It is also part of chemotherapeutic regimens against various types of human cancers, including HNSCC (32-34). Solid tumors that developed resistance to VEGF or vascular endothelial growth factor receptor (VEGFR)-targeted therapy with bevacizumab showed sensitivity towards mTOR inhibitors (32). mTOR is an integration center for several intracellular signaling pathways frequently dysregulated in cancer (26, 33). Synergistic effects of mTOR inhibitors with other targeted therapy drugs and radiochemotherapy illustrate the integrative function of mTOR (35-37). Lymph node metastases of HNSCC often show activated mTOR signaling. Its inhibition with everolimus reduces lymphangiogenesis at the primary tumor site and prevents lymphatic dissemination (38).

Sorafenib and sunitinib are multi-tyrosine kinase inhibitors. Both drugs target VEGFR and platelet-derived growth factor receptor (PDGFR). Furthermore, sorafenib inhibits Raf kinases, while sunitinib inhibits c-KIT, RET and colony stimulating factor (CSF). Sorafenib was applied in the therapy of hepatocellular, thyroid and renal cell carcinoma, lung cancer, melanoma and HNSCC (39-42). The combination of sorafenib with other targeted anticancer drugs, chemotherapy and radiation led to promising synergistic effects (43, 44).

Sunitinib was established in treatment regimens for gastrointestinal stromal tumors (GIST), renal cell carcinoma, pancreatic neuroendocrine tumors, breast, lung, thyroid and colorectal cancers (45, 46). Recently, it was evaluated for HNSCC therapy in clinical trials (47-49).

The application of everolimus, sorafenib and sunitinib in HNSCC therapy has been evaluated in several clinical trials (36, 37, 47-52); however, none of these studies took into account the HPV status of SCC (squamous cell carcinoma) cells. Our study evaluated the protein levels of mTOR and AREG in HPV-positive (CERV196) and HPV-negative (HNSCC 11A and 14C) SCC and their alteration following incubation with everolimus, sorafenib and sunitinib. As far as we are aware of, this is one of the first *in vitro* studies to investigate the impact of these small-molecule drugs on SCC, particularly with regard to HPV-dependent differences.

Materials and Methods

Cell lines. The human cell lines HNSCC 11A and 14C were received from Dr. T.E. Carey (University of Michigan, Ann Arbor, MI, USA) and originated from a SCC of the oropharynx and larynx. CERV196 cells were obtained from a p16-positive SCC of the uterine cervix (CLS, Eppelheim, Germany). Dulbecco's modified essential medium (DMEM) (Fisher Scientific and Co., Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies Inc., Gaithersburg, MD, USA) was used as medium for HNSCC 11A and 14C. CERV196 cells were kept in Eagle's minimum essential medium with 2 mM L-glutamine, 10% FCS and Earle's balanced salt solution (BSS) with 1.5 g/l sodium bicarbonate, 0.1 mM amino acids and 1.0 mM sodium pyruvate. Cell cultures were carried out at 37˚C in a fully humidified atmosphere with 5% CO2. Everolimus (Novartis, Basel, Switzerland), sorafenib (Bayer, Leverkusen, Germany) and sunitinib (Pfizer, New York, NY, USA) were made up in dimethylsulfoxide (DMSO) at the time of use. The cells were exposed to drug concentrations ranging from 1.0-25.0 μmol/ml for 2 to 8 days. These drug concentrations were chosen after the alamarBlue (AbD

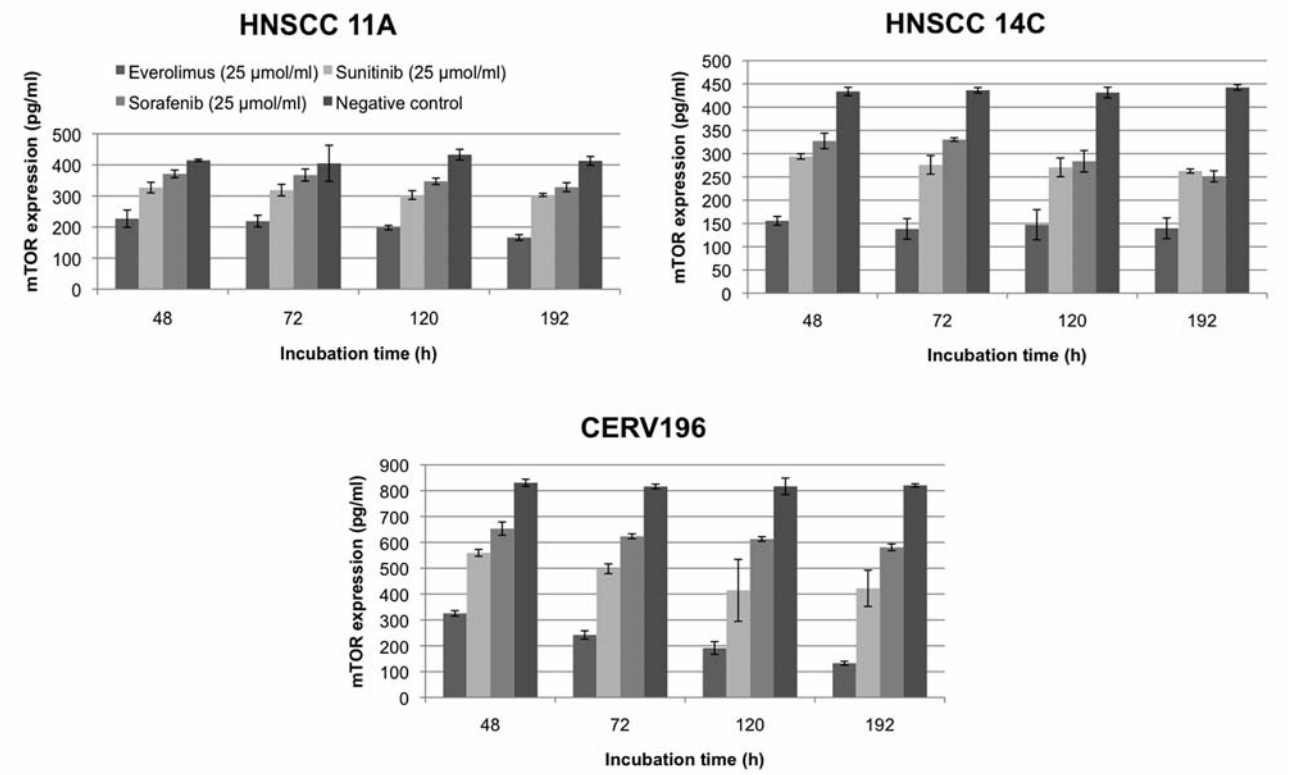

Figure 1. *mTOR expression in HNSCC 11A, 14C and CERV196 after incubation with everolimus, sunitinib or sorafenib compared to the negative control. Data are mean values. Standard deviation is indicated.*

Serotec, Oxford, UK) cell proliferation assay was performed, which measured the proliferation of HNSCC tumor cell lines quantitatively and indicated the relative cytotoxicity of the studied drugs.

Enzyme-linked immunosorbent assay (ELISA) for total mTOR and AREG. Following incubation, cells were washed with phosphate buffered saline (PBS). Then, 350 µl of lysis buffer were added to each well. The lysed cells were stirred up with a vortex at 2-8˚C for 30 min and microcentrifuged with 5,000×g for 5 min. The supernatant was pipetted into a clean tube.

Protein concentrations were determined with the ELISA technique (R&D Systems, Wiesbaden, Germany): DuoSet IC Human phosphorylated mTOR (DYC1665) and DuoSet Human Amphiregulin (AREG) (DY262).

The sandwich ELISA system used a solid-phase capture antibody specific for either human phosphorylated mTOR or AREG and a specific detection antibody using a standard streptavidin-horseradish peroxidase (HRP) format specific for phosphorylated mTOR or AREG. The capture antibody was diluted to its working concentration (2 µg/ml for both mTOR and AREG). Then, 100 µl of the capture antibody were added to each well and incubated overnight. The contents of each well were aspirated and the wells were washed three times with 400 µl of Tween buffer. Subsequently, 300 µl of block buffer were added to each well and incubated for 1-2 h followed by another Tween buffer wash as described above. The detection antibody was diluted to its working concentration (1 µg/ml for mTOR and 2 µg/ml for AREG) and 100 µl of the detection antibody were added to each well for 2 h. The wells were washed again as described above and 100 µl of streptavidin-HRP (diluted according to the manufacturer's instructions) were added to each well followed by 20 min of incubation at room temperature. Afterwards, the wells were washed as described above and 100 µl of substrate solution were added to each well for 20 min followed by 50 µl of stop solution. According to the manufacturer's directions, each ELISA was performed with 100 µl of supernatant. All analyses and calibrations were carried out three times. The calibrations on each microtiter plate included recombinant human mTOR and AREG standards that were provided in the kits. Optical density was measured using a microplate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm, and concentrations were reported as pg/ml. The range of detection for mTOR was 156-10,000 pg/ml and 15.6-1,000 pg/ml for AREG. The interassay coefficient of variation reported by the manufacturer was below 10%.

Measuring total protein. Total protein was measured with the DC Protein Assay (BioRad, Hercules, CA, USA). Cells were incubated, lysed and centrifuged as previously described. Dilutions of protein standard were prepared according to the manufacturer's instructions. Measurement was performed on 100 µl of protein standard or cell supernatant with a spectrophotometer set to 750 nm; concentrations were reported as µg/ml.

Table I. *ELISA of mTOR expression in HNSCC 11A, 14C and CERV196 after incubation with everolimus, sunitinib or sorafenib compared to the negative control (statistical significance is shown in bold).*

Incubation time (h)	Negative control	Everolimus (25 μmol/ml)		Sunitinib (25 μmol/ml)		Sorafenib (25 μmol/ml)	
	Mean value	Mean value	*p*-Value	Mean value	*p*-Value	Mean value	*p*-Value
HNSCC 11A							
48	414.667	227	0.762	327	0.090	371	**0.072**
72	405	219	0.500	319	**0.021**	367.333	**0.015**
120	433	198.333	0.808	303	**0.003**	347.333	**0.055**
192	413	166.333	0.615	303.333	**0.025**	328.333	**0.038**
HNSCC 14C							
48	433.667	155.667	**0.004**	294	**0.003**	327.333	0.491
72	436.333	138.333	**0.017**	276	0.207	330.333	0.695
120	431.333	147.333	**0.039**	270.667	0.336	283.667	0.331
192	442.333	139.667	**0.009**	262.667	0.659	251.333	0.427
CERV 196							
48	830.667	325.333	0.113	559.333	**0.011**	653.333	**0.006**
72	816	242	0.243	498	**0.002**	623.667	**0.007**
120	816.667	191.333	0.365	414.333	**0.002**	613	**0.008**
192	820.333	132.667	0.563	422.333	**<0.0001**	581	**0.024**

Statistical analysis. Statistical analysis was performed in cooperation with PD Dr. C. Weiss, Institute of Biomathematics, Faculty of Medicine, Mannheim, Germany. A p-value ≤0.05 was considered statistically significant. The two-coefficient variance test (SAS Statistics, Cary, NC, USA) and Dunnett's test were used.

Results

Total protein assay. Total protein levels were evaluated in order to differentiate a decline of AREG and mTOR protein expression from cytotoxicity of the applied drugs. The total protein level of the cell lysate was compared to the protein level of the target values. We detected a slight increase of the protein quotient (expression of the target value/total protein level) for HNSCC 11A and 14C after incubation with sunitinib and sorafenib in a time-dependent manner, which could be evidence of increased apoptosis. A similar effect was not observed in p16-positive CERV196. Everolimus did not increase protein quotient (data not shown).

ELISA for mTOR expression in HNSCC 14C, 11A and CERV 196 cells. mTOR was expressed in all three cell lines. Expression levels of mTOR in CERV196 were twice as high compared to p16-negative HNSCC 11A and 14C. In CERV 196, sunitinib and sorafenib significantly decreased mTOR expression independent of the incubation time. The p-values for sunitinib varied from <0.001-0.002 and for sorafenib from 0.006-0.024. In HNSCC 11A, sunitinib suppressed mTOR expression significantly after 3, 5 and 8 days of incubation (p=0.003-0.025). Similar results were observed for sorafenib in HNSCC 11A with a significant decrease of mTOR expression after 2, 3 and 8 days of incubation (p=0.015-0.072). In HNSCC 14C, neither sunitinib nor sorafenib led to a statistically significant reduction of mTOR expression with the exception of sunitinib after 2 days of incubation (p=0.003). Although everolimus considerably decreased mTOR expression in CERV196 and HNSCC 11A in an incubation-time dependent manner, the reduction was not statistically significant. On the contrary, we detected significant suppression of mTOR expression by everolimus in HNSCC 14C independent of the incubation time (p=0.009-0.039). Increasing drug concentrations had no statistically significant effect on mTOR expression. For simplification, only the data for 25 μmol/ml are show in Table I and Figure 1.

ELISA of AREG expression in HNSCC 14C, 11A and CERV196. Expression of AREG was detected in all three cell lines. In CERV196, expression levels were considerably lower (reduced by almost 50%) compared to HNSCC 11A and 14C. Both sunitinib and sorafenib significantly suppressed AREG in CERV196. Sunitinib almost completely abolished the expression of AREG in CERV196 compared to the chemonaïve control with p-values between 0.001 and 0.005. Sorafenib displayed a statistically significant impact on AREG expression in CERV196 (p<0.001). Everolimus significantly suppressed AREG in CERV196 (p=0.014-0.026), with one exception after 120 h of incubation (p=0.229). The impact of everolimus on HNSCC 11A and 14C was much smaller. Significant

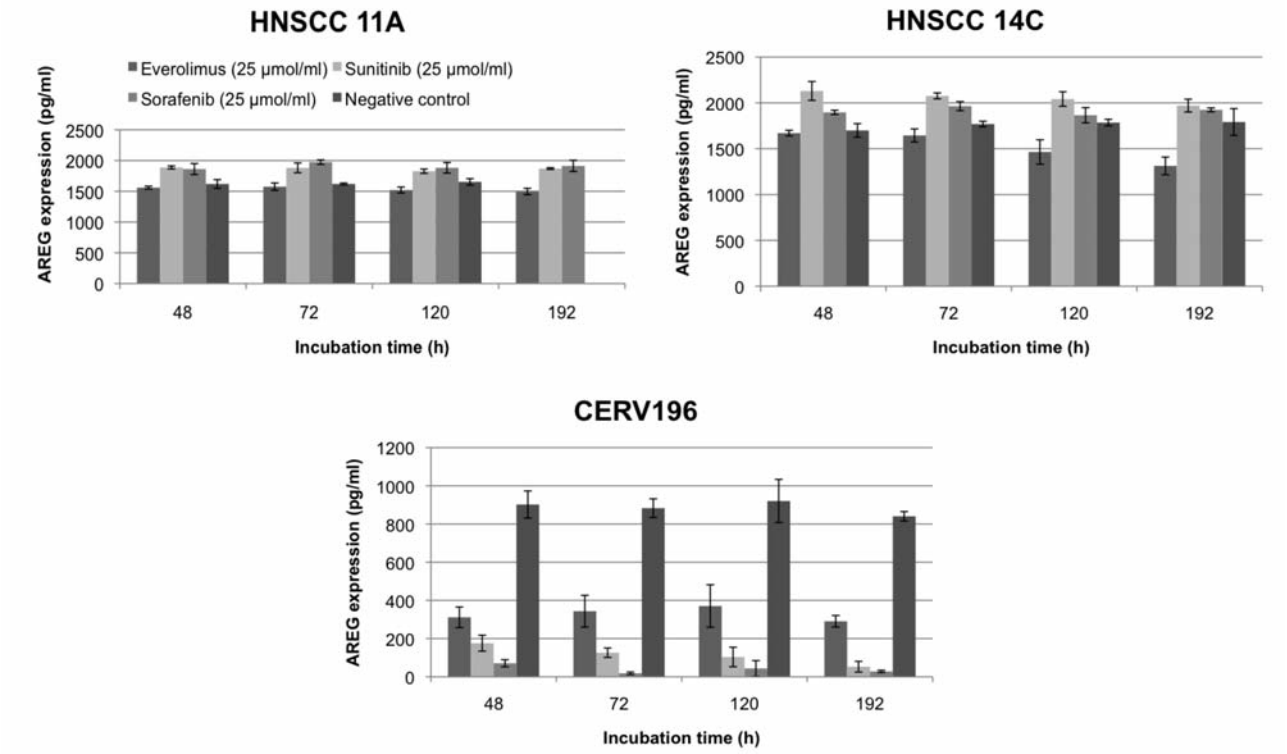

Figure 2. *AREG expression in HNSCC 11A, 14C and CERV196 after incubation with everolimus, sunitinib or sorafenib compared to the negative control. Data are mean values. Standard deviation is indicated.*

suppression of AREG could be detected after 72 h of incubation in HNSCC 11A only (p=0.019). Due to technical difficulties, the sample for the negative control after 192 h of incubation could not be analyzed. Consequently, we were unable to determine a p-value for this sample. In HNSCC 14C, we detected a significant alteration of AREG levels under incubation with everolimus after 48 h and 72 h (p<0.001 and 0.048, respectively). Interestingly, both multi-tyrosine kinase inhibitors sunitinib and sorafenib significantly increased the expression of AREG in HNSCC 14C and 11A independent of the incubation time (p≤0.008). Drug concentrations of 10 µmol/ml or higher were significantly more effective at altering protein expression. For simplification, only the data for 25 µmol/ml are show in Table II and Figure 2.

Discussion

The formation of a vasculature is a crucial step for sustained tumor growth, progression, invasion and migration of tumor cells, which eventually leads to lymph node or distant metastasis. Tumor cells express a set of various angiogenic factors that facilitate the formation of new blood vessels. Therefore, it is reasonable to target pro-angiogenic factors in anticancer therapy in order to prevent or decelerate the aforementioned process. Riedel *et al*. demonstrated a correlation between the microvessel density in a tumor and the recurrence or metastasis of a tumor (53, 54). A set of angiogenic factors either released by the tumor or the stroma regulate the process of vascularization and neoangiogenesis (55, 56).

This is one of the first *in vitro* studies to investigate the impact of everolimus, sunitinib and sorafenib on the expression of AREG and mTOR in p16-positive and -negative SCC. Our results showed that both AREG and mTOR are expressed in all three cell lines. We found that the expression of AREG in CERV196 is considerably lower (reduced by almost 50%) when compared to HNSCC 11A and 14C. Tinhofer *et al*. reported that HNSCC patients with high expression levels of EGFR and AREG are less likely to benefit from a combination therapy with cetuximab and docetaxel (57). Thus, our findings support the hypothesis that HPV positivity in HNSCC is a rather favorable factor concerning prognosis and outcome for the patient. On the contrary, expression levels for mTOR were considerably higher in CERV196 when compared to HNSCC 11A and 14C, which implies increased activity of mTOR signaling pathways in CERV196.

Sunitinib and sorafenib suppressed AREG expression in CERV196 independently of the incubation time, which could lead to an anti-angiogenic effect and delay tumor progression and dissemination. Interestingly, sunitinib almost completely abolished AREG expression in an incubation time-dependent

Table II. *ELISA of AREG expression in HNSCC 11A, 14C and CERV196 after incubation with everolimus, sunitinib or sorafenib compared to the negative control (statistical significance is shown in bold).*

Incubation time (h)	Negative control	Everolimus (25 μmol/ml)		Sunitinib (25 μmol/ml)		Sorafenib (25 μmol/ml)	
	Mean value	Mean value	*p*-Value	Mean value	*p*-Value	Mean value	*p*-Value
HNSCC 11A							
48	1619.333	1559.333	0.096	1888	**0.001**	1861.667	**<0.001**
72	1619.333	1575.333	**0.019**	1881	**<0.001**	1974	**0.001**
120	1653.333	1520.667	0.141	1826.667	**0.003**	1882	**<0.001**
192	-	1497	-	1870.333	-	1912.667	-
HNSCC 14C							
48	1699.333	1670.333	**<0.001**	2130	**0.007**	1896.667	**0.008**
72	1769.333	1644.667	**0.048**	2076	**0.001**	1964.667	**<0.001**
120	1785.333	1464.667	0.215	2042	**<0.001**	1865.333	**<0.001**
192	1791.667	1313	0.103	1970.667	**0.002**	1924	**<0.001**
CERV 196							
48	902	311.667	**0.026**	176	**0.003**	653.333	**<0.001**
72	883	343.333	**0.023**	126.333	**0.005**	623.667	**<0.001**
120	920.333	370.667	0.229	104	**0.001**	613	**<0.001**
192	840.333	290.667	**0.014**	53	**0.001**	581	**<0.001**

manner. In HNSCC 11A and 14C, only a slight reduction of AREG expression could be detected after incubation with everolimus. Surprisingly, we detected increased expression levels of AREG in HNSCC 11A and 14C after incubation with sunitinib and sorafenib irrespective of the incubation time. Sunitinib and sorafenib are inhibitors of VEGFR and PDGFR but not EGFR, the receptor of AREG. As previously mentioned, EGFR is over-expressed in 42-80% of HNSCC (18-21). An up-regulation of AREG expression following exposure to sunitinib and sorafenib could be explained as an evasive mechanism for VEGFR and PDGFR inhibition by an autocrine pro-angiogenic stimulus *via* AREG and EGFR. This is distinct from a mechanism of drug resistance because inhibition of the targets VEGFR and PDGFR is still possible but the anti-angiogenic potency of the multi-tyrosine kinase inhibitors is compromised. It is also a possible explanation why a monotherapy with sunitinib or sorafenib is not sufficient for HNSCC therapy and should be used in a combination therapy (47-51). Interestingly, p16-positive CERV196 did not increase AREG expression under sunitinib and sorafenib. Furthermore, expression levels of AREG in the chemonaïve control of CERV196 were remarkably lower when compared to HNSCC 11A and 14C. This could be evidence that p16-positive CERV196 has higher susceptibility towards a targeted anti-angiogenic therapy with sunitinib and sorafenib as already reported for PDGFR (58).

As previously described, mTOR integrates the signaling cascades of several messengers, including EGFR, and induces angiogenesis *via* increased protein synthesis of, for instance, VEGF (25, 29, 30). mTOR inhibition has been shown to increase cetuximab sensitivity in EGFR-resistant HNSCC (59). Hirashima and colleagues reported that the mTOR pathway is aberrantly activated in many SCCs (60). We demonstrated that sunitinib and sorafenib significantly decrease mTOR expression apart from their main mechanism of action. Consequently, we have to state a pleiotropic effect of these two drugs. Sunitinib appeared to be more effective compared to sorafenib; however, this difference was not significant. Again, p16-positive CERV196 was more susceptible towards a reduction of mTOR compared to HNSCC 11A and 14C. Everolimus reduced mTOR expression in all cell lines. Surprisingly, the effect of everolimus in HNSCC 11A and CERV196 did not reach significance. Reduced expression of mTOR would lead to an impaired PI3K/Akt signaling pathway and increase the expression of the tumor suppressor proteins PTEN (phosphatase and tensin homolog deleted on chromosome 10) and p53 (27, 61). Patel and colleagues reported a decreased formation of lymphatic metastasis under mTOR inhibition in HNSCC (38).

As previously mentioned, the protein quotient (expression of the target value/total protein level) increased under incubation with sunitinib and sorafenib in an incubation time-dependent manner in HNSCC 11A and 14C but not in CERV196. Saito *et al*. reported that sunitinib induces apoptosis *via* a VEGFR2/Akt/mTOR pathway (62). The fact that CERV196 cells showed higher expression levels of mTOR even under incubation with sunitinib and sorafenib when compared to the chemonaïve control of HNSCC 11A and 14C might explain why a similar apoptotic effect of

sunitinib and sorafenib is missing in CERV196. Interestingly, we found no signs of increased apoptosis after incubation with everolimus.

Our results confirm that a targeted monotherapy may lead to evasive mechanisms within cellular signaling. CERV196 was characterized by an increased susceptibility towards AREG suppression under everolimus, sorafenib and sunitinib. Further studies are mandatory to investigate HPV-dependent differences in therapy response and possible consequences for treatment regimens in the future.

Acknowledgements

The Authors would like to thank Petra Prohaska for her outstanding technical assistance and PD Dr. C. Weiss for her distinguished advice in statistical analysis.

References

1 Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer *136*: E359-386, 2015.

2 Edwards BK, Brown ML, Wingo PA, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN and Pickle LW: Annual report to the nation on the status of cancer, 1975-2002, featuring population-based trends in cancer treatment. J Natl Cancer Inst *97*: 1407-1427, 2005.

3 Hashibe M, Brennan P, Chuang SC, Boccia S, Castellsague X, Chen C, Curado MP, Dal Maso L, Daudt AW, Fabianova E, Fernandez L, Wunsch-Filho V, Franceschi S, Hayes RB, Herrero R, Kelsey K, Koifman S, La Vecchia C, Lazarus P, Levi F, Lence JJ, Mates D, Matos E, Menezes A, McClean MD, Muscat J, Eluf-Neto J, Olshan AF, Purdue M, Rudnai P, Schwartz SM, Smith E, Sturgis EM, Szeszenia-Dabrowska N, Talamini R, Wei Q, Winn DM, Shangina O, Pilarska A, Zhang ZF, Ferro G, Berthiller J and Boffetta P: Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Cancer Epidemiol Biomarkers Prev *18*: 541-550, 2009.

4 Hashibe M, Brennan P, Benhamou S, Castellsague X, Chen C, Curado MP, Dal Maso L, Daudt AW, Fabianova E, Fernandez L, Wunsch-Filho V, Franceschi S, Hayes RB, Herrero R, Koifman S, La Vecchia C, Lazarus P, Levi F, Mates D, Matos E, Menezes A, Muscat J, Eluf-Neto J, Olshan AF, Rudnai P, Schwartz SM, Smith E, Sturgis EM, Szeszenia-Dabrowska N, Talamini R, Wei Q, Winn DM, Zaridze D, Zatonski W, Zhang ZF, Berthiller J and Boffetta P: Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. J Natl Cancer Inst *99*: 777-789, 2007.

5 Sturgis EM and Cinciripini PM: Trends in head and neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers? Cancer *110*: 1429-1435, 2007.

6 Shiboski CH, Schmidt BL and Jordan RC: Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20-44 years. Cancer *103*: 1843-1849, 2005.

7 Chaturvedi AK, Engels EA, Anderson WF and Gillison ML: Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. J Clin Oncol *26*: 612-619, 2008.

8 Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S and Viscidi R: Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst *100*: 407-420, 2008.

9 Gillison ML and Lowy DR: A causal role for human papillomavirus in head and neck cancer. Lancet *363*: 1488-1489, 2004.

10 Dayyani F, Etzel CJ, Liu M, Ho CH, Lippman SM and Tsao AS: Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC). Head Neck Oncol *2*: 15, 2010.

11 Kreimer AR, Clifford GM, Boyle P and Franceschi S: Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev *14*: 467-475, 2005.

12 Gillison ML: Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. Semin Oncol *31*: 744-754, 2004.

13 Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, Ahrlund-Richter S, Marklund L, Romanitan M, Lindquist D, Ramqvist T, Lindholm J, Sparen P, Ye W, Dahlstrand H, Munck-Wikland E and Dalianis T: Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? Int J Cancer *125*: 362-366, 2009.

14 Scheffner M, Werness BA, Huibregtse JM, Levine AJ and Howley PM: The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell *63*: 1129-1136, 1990.

15 Rautava J, Kuuskoski J, Syrjanen K, Grenman R and Syrjanen S: HPV genotypes and their prognostic significance in head and neck squamous cell carcinomas. J Clin Virol

16 Psyrri A, Seiwert TY and Jimeno A: Molecular pathways in head and neck cancer: EGFR, PI3K, and more. Am Soc Clin Oncol Educ Book 246-255, 2013.

17 Pentheroudakis G, Kotoula V, De Roock W, Kouvatseas G, Papakostas P, Makatsoris T, Papamichael D, Xanthakis I, Sgouros J, Televantou D, Kafiri G, Tsamandas AC, Razis E, Galani E, Bafaloukos D, Efstratiou I, Bompolaki I, Pectasides D, Pavlidis N, Tejpar S and Fountzilas G: Biomarkers of benefit from cetuximab-based therapy in metastatic colorectal cancer: interaction of EGFR ligand expression with RAS/RAF, PIK3CA genotypes. BMC Cancer *13*: 49, 2013.

18 Sheu JJ, Hua CH, Wan L, Lin YJ, Lai MT, Tseng HC, Jinawath N, Tsai MH, Chang NW, Lin CF, Lin CC, Hsieh LJ, Wang TL, Shih Ie M and Tsai FJ: Functional genomic analysis identified epidermal growth factor receptor activation as the most common genetic event in oral squamous cell carcinoma. Cancer Res *69*: 2568-2576, 2009.

19 Kim S, Grandis JR, Rinaldo A, Takes RP and Ferlito A: Emerging perspectives in epidermal growth factor receptor targeting in head and neck cancer. Head Neck *30*: 667-674, 2008.

20 Ongkeko WM, Altuna X, Weisman RA and Wang-Rodriguez J: Expression of protein tyrosine kinases in head and neck squamous cell carcinomas. Am J Clin Pathol *124*: 71-76, 2005.

21 Grandis JR and Tweardy DJ: Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res *53*: 3579-3584, 1993.

22 Temam S, Kawaguchi H, El-Naggar AK, Jelinek J, Tang H, Liu DD, Lang W, Issa JP, Lee JJ and Mao L: Epidermal growth factor receptor copy number alterations correlate with poor clinical outcome in patients with head and neck squamous cancer. J Clin Oncol *25*: 2164-2170, 2007.

23 Zimmermann M, Zouhair A, Azria D and Ozsahin M: The epidermal growth factor receptor (EGFR) in head and neck cancer: its role and treatment implications. Radiat Oncol *1*: 11, 2006.

24 Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL, Freilino ML, Graner MW, Wikstrand CJ, Bigner DD, Gooding WE, Furnari FB and Grandis JR: Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clin Cancer Res *12*: 5064-5073, 2006.

25 Lionello M, Lovato A, Staffieri A, Blandamura S, Turato C, Giacomelli L, Staffieri C and Marioni G: The EGFR-mTOR pathway and laryngeal cancer angiogenesis. Eur Arch Otorhinolaryngol *271*: 757-764, 2014.

26 Dunlop EA and Tee AR: Mammalian target of rapamycin complex 1: signalling inputs, substrates and feedback mechanisms. Cell Signal *21*: 827-835, 2009.

27 Ocana A, Vera-Badillo F, Al-Mubarak M, Templeton AJ, Corrales-Sanchez V, Diez-Gonzalez L, Cuenca-Lopez MD, Seruga B, Pandiella A and Amir E: Activation of the PI3K/ mTOR/AKT pathway and survival in solid tumors: systematic review and metaanalysis. PLoS One *9*: e95219, 2014.

28 Dienstmann R, Rodon J, Serra V and Tabernero J: Picking the point of inhibition: a comparative review of PI3K/AKT/mTOR pathway inhibitors. Mol Cancer Ther *13*: 1021-1031, 2014.

29 Humar R, Kiefer FN, Berns H, Resink TJ and Battegay EJ: Hypoxia enhances vascular cell proliferation and angiogenesis *in vitro via* rapamycin (mTOR)-dependent signaling. FASEB J *16*: 771-780, 2002.

30 Karar J and Maity A: PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci *4*: 51, 2011.

31 Wang SS, Chou NK, Chi NH, Huang SC, Wu IH, Wang CH, Yu HY, Chen YS, Tsao CI, Ko WJ and Shun CT: Clinical experience of tacrolimus with everolimus in heart transplantation. Transplant Proc *44*: 907-909, 2012.

32 Agarwala SS and Case S: Everolimus (RAD001) in the treatment of advanced renal cell carcinoma: a review. Oncologist *15*: 236-245, 2010.

33 Figlin RA, Brown E, Armstrong AJ, Akerley W, Benson AB, 3rd, Burstein HJ, Ettinger DS, Febbo PG, Fury MG, Hudes GR, Kies MS, Kwak EL, Morgan RJ, Jr., Mortimer J, Reckamp K, Venook AP, Worden F and Yen Y: NCCN Task Force Report: mTOR inhibition in solid tumors. J Natl Compr Canc Netw 6 *Suppl 5*: S1-20; quiz S21-22, 2008.

34 Nguyen SA, Walker D, Gillespie MB, Gutkind JS and Day TA: mTOR inhibitors and its role in the treatment of head and neck squamous cell carcinoma. Curr Treat Options Oncol *13*: 71-81, 2012.

35 Bozec A, Etienne-Grimaldi MC, Fischel JL, Sudaka A, Toussan N, Formento P and Milano G: The mTOR-targeting drug temsirolimus enhances the growth-inhibiting effects of the cetuximab-bevacizumab-irradiation combination on head and neck cancer xenografts. Oral Oncol *47*: 340-344, 2011.

36 Bozec A, Sudaka A, Toussan N, Fischel JL, Etienne-Grimaldi MC and Milano G: Combination of sunitinib, cetuximab and irradiation in an orthotopic head and neck cancer model. Ann Oncol *20*: 1703-1707, 2009.

37 Fury MG, Sherman E, Haque S, Korte S, Lisa D, Shen R, Wu N and Pfister D: A phase I study of daily everolimus plus low-dose weekly cisplatin for patients with advanced solid tumors. Cancer Chemother Pharmacol *69*: 591-598, 2012.

38 Patel V, Marsh CA, Dorsam RT, Mikelis CM, Masedunskas A, Amornphimoltham P, Nathan CA, Singh B, Weigert R, Molinolo AA and Gutkind JS: Decreased lymphangiogenesis and lymph node metastasis by mTOR inhibition in head and neck cancer. Cancer Res *71*: 7103-7112, 2011.

39 Sawicki M, Szudy A, Szczyrek M, Krawczyk P and Klatka J: Molecularly targeted therapies in head and neck cancers. Otolaryngol Pol *66*: 307-312, 2012.

40 Adjei AA, Blumenschein GR, Jr., Mandrekar S, Hillman S, Gatzemeier U and Heigener D: Long-term safety and tolerability of sorafenib in patients with advanced non-small-cell lung cancer: a case-based review. Clin Lung Cancer *12*: 212-217, 2011.

41 Du J, Qian X and Liu B: Long-term progression-free survival in a case of hepatocellular carcinoma with vertebral metastasis treated with a reduced dose of sorafenib: Case report and review of the literature. Oncol Lett *5*: 381-385, 2013.

42 Egberts F, Gutzmer R, Ugurel S, Becker JC, Trefzer U, Degen A, Schenck F, Frey L, Wilhelm T, Hassel JC, Schadendorf D, Livingstone E, Mauch C, Garbe C, Berking C, Rass K, Mohr P, Kaehler KC, Weichenthal M and Hauschild A: Sorafenib and pegylated interferon-alpha2b in advanced metastatic melanoma: a multicenter phase II DeCOG trial. Ann Oncol *22*: 1667-1674, 2011.

43 Ibrahim N, Yu Y, Walsh WR and Yang JL: Molecular targeted therapies for cancer: sorafenib mono-therapy and its combination with other therapies (review). Oncol Rep 27: 1303-1311, 2012.

44 Dal Lago L, D'Hondt V and Awada A: Selected combination therapy with sorafenib: a review of clinical data and perspectives in advanced solid tumors. Oncologist *13*: 845-858, 2008.

45 Blumenschein GR, Jr., Ciuleanu T, Robert F, Groen HJ, Usari T, Ruiz-Garcia A, Tye L, Chao RC and Juhasz E: Sunitinib plus erlotinib for the treatment of advanced/metastatic non-small-cell lung cancer: a lead-in study. J Thorac Oncol *7*: 1406-1416, 2012.

46 Pasqualetti G, Ricci S, Dardano A, Ferdeghini M, Del Tacca M and Monzani F: The emerging role of sunitinib in the treatment of advanced epithelial thyroid cancer: our experience and review of literature. Mini Rev Med Chem *11*: 746-752, 2011.

47 Choong NW, Kozloff M, Taber D, Hu HS, Wade J, 3rd, Ivy P, Karrison TG, Dekker A, Vokes EE and Cohen EE: Phase II study of sunitinib malate in head and neck squamous cell carcinoma. Invest New Drugs *28*: 677-683, 2010.

48 Fountzilas G, Fragkoulidi A, Kalogera-Fountzila A, Nikolaidou M, Bobos M, Calderaro J, Andreiuolo F and Marselos M: A phase II study of sunitinib in patients with recurrent and/or metastatic non-nasopharyngeal head and neck cancer. Cancer Chemother Pharmacol *65*: 649-660, 2010.

49 Machiels JP, Henry S, Zanetta S, Kaminsky MC, Michoux N, Rommel D, Schmitz S, Bompas E, Dillies AF, Faivre S, Moxhon A, Duprez T and Guigay J: Phase II study of sunitinib in recurrent or metastatic squamous cell carcinoma of the head and neck: GORTEC 2006-01. J Clin Oncol *28*: 21-28, 2010.

50 Elser C, Siu LL, Winquist E, Agulnik M, Pond GR, Chin SF, Francis P, Cheiken R, Elting J, McNabola A, Wilkie D, Petrenciuc O and Chen EX: Phase II trial of sorafenib in patients with recurrent or metastatic squamous cell carcinoma of the head and neck or nasopharyngeal carcinoma. J Clin Oncol *25*: 3766-3773, 2007.

51 Williamson SK, Moon J, Huang CH, Guaglianone PP, LeBlanc M, Wolf GT and Urba SG: Phase II evaluation of sorafenib in advanced and metastatic squamous cell carcinoma of the head and neck: Southwest Oncology Group Study S0420. J Clin Oncol *28*: 3330-3335, 2010.

52 Fury MG, Sherman E, Ho AL, Xiao H, Tsai F, Nwankwo O, Sima C, Heguy A, Katabi N, Haque S and Pfister DG: A phase 1 study of everolimus plus docetaxel plus cisplatin as induction chemotherapy for patients with locally and/or regionally advanced head and neck cancer. Cancer *119*: 1823-1831, 2013.

53 Riedel F, Gotte K, Schwalb J, Schafer C and Hormann K: Vascular endothelial growth factor expression correlates with p53 mutation and angiogenesis in squamous cell carcinoma of the head and neck. Acta Otolaryngol *120*: 105-111, 2000.

54 Riedel F, Gotte K, Schwalb J, Wirtz H, Bergler W and Hormann K: Serum levels of vascular endothelial growth factor in patients with head and neck cancer. Eur Arch Otorhinolaryngol *257*: 332-336, 2000.

55 Tlsty TD and Hein PW: Know thy neighbor: stromal cells can contribute oncogenic signals. Curr Opin Genet Dev *11*: 54-59, 2001.

56 De Wever O and Mareel M: Role of tissue stroma in cancer cell invasion. J Pathol *200*: 429-447, 2003.

57 Tinhofer I, Klinghammer K, Weichert W, Knodler M, Stenzinger A, Gauler T, Budach V and Keilholz U: Expression of amphiregulin and EGFRvIII affect outcome of patients with squamous cell carcinoma of the head and neck receiving cetuximab-docetaxel treatment. Clin Cancer Res *17*: 5197-5204, 2011.

58 Aderhold C, Faber A, Grobschmidt GM, Chakraborty A, Bockmayer A, Umbreit C, Birk R, Stern-Straeter J, Hormann K and Schultz JD: Small Molecule-based Chemotherapeutic Approach in p16-positive and -negative HNSCC In Vitro. Anticancer Res *33*: 5385-5393, 2013.

59 D'Amato V, Rosa R, D'Amato C, Formisano L, Marciano R, Nappi L, Raimondo L, Di Mauro C, Servetto A, Fusciello C, Veneziani BM, De Placido S and Bianco R: The dual PI3K/mTOR inhibitor PKI-587 enhances sensitivity to cetuximab in EGFR-resistant human head and neck cancer models. Br J Cancer *110*: 2887-2895, 2014.

60 Hirashima K, Baba Y, Watanabe M, Karashima RI, Sato N, Imamura Y, Nagai Y, Hayashi N, Iyama KI and Baba H: Aberrant activation of the mTOR pathway and anti-tumour effect of everolimus on oesophageal squamous cell carcinoma. Br J Cancer *106*: 876-882, 2012.

61 Herzog A, Bian Y, Vander Broek R, Hall B, Coupar J, Cheng H, Sowers AL, Cook JD, Mitchell JB, Chen Z, Kulkarni AB and Van Waes C: PI3K/mTOR inhibitor PF-04691502 antitumor activity is enhanced with induction of wild-type TP53 in human xenograft and murine knockout models of head and neck cancer. Clin Cancer Res *19*: 3808-3819, 2013.

62 Saito Y, Tanaka Y, Aita Y, Ishii KA, Ikeda T, Isobe K, Kawakami Y, Shimano H, Hara H and Takekoshi K: Sunitinib induces apoptosis in pheochromocytoma tumor cells by inhibiting VEGFR2/Akt/mTOR/S6K1 pathways through modulation of Bcl-2 and BAD. Am J Physiol Endocrinol Metab *302*: E615-625, 2012.

Received December 14, 2014
Revised February 7, 2015
Accepted February 10, 2015