Title

Prevention of tumor growth driven by PIK3CA and HPV oncogenes by targeting mTOR signaling with metformin in oral squamous carcinomas expressing OCT3.

Journal

Cancer Prevention Research, 8(3)

Authors

Madera, Dmitri

Vitale-Cross, Lynn

Martin, Daniel

et al.

Publication Date

2015-03-01

DOI

10.1158/1940-6207.CAPR-14-0348

Peer reviewed

Published in final edited form as:

Cancer Prev Res (Phila). 2015 March ; 8(3): 197–207. doi:10.1158/1940-6207.CAPR-14-0348.

Prevention of tumor growth driven by *PIK3CA* and HPV oncogenes by targeting mTOR signaling with metformin in oral squamous carcinomas expressing OCT3

Dmitri Madera[1], **Lynn Vitale-Cross**[1], **Daniel Martin**[1], **Abraham Schneider**[2], **Alfredo A. Molinolo**[1], **Nitin Gangane**[3], **Thomas E. Carey**[4], **Jonathan B. McHugh**[4], **Christine M. Komarck**[4], **Heather M. Walline**[4], **William N. William Jr.**[5], **Raja R. Seethala**[6], **Robert Ferris**[7], and **J. Silvio Gutkind**[1,*]

[1]Molecular Carcinogenesis Section, Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland [2]Department of Oncology and Diagnostic Sciences, School of Dentistry and Greenebaum Cancer Center, Program in Oncology, University of Maryland, Baltimore, Maryland [3]Department of Pathology, Mahatma Gandhi Institute of Medical Sciences, Wardha, Maharashtra, India [4]Department of Otolaryngology-Head Neck Surgery, and the Head and Neck SPORE Tissue Core, University of Michigan, Ann Arbor, Michigan [5]Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas [6]University of Pittsburgh School of Medicine, Pathology Program, Pittsburgh, Philadelphia [7]University of Pittsburgh School of Medicine, Otolaryngology, Immunology, Cancer Immunology Program, Pittsburgh, Philadelphia

Abstract

Most head and neck squamous cell carcinomas (HNSCC) exhibit a persistent activation of the PI3K-mTOR signaling pathway. We have recently shown that metformin, an oral antidiabetic drug that is also used to treat lipodystrophy in HIV-infected (HIV+) individuals, diminishes mTOR activity and prevents the progression of chemically-induced experimental HNSCC premalignant lesions. Here, we explored the preclinical activity of metformin in HNSCCs harboring *PIK3CA* mutations and *HPV* oncogenes, both representing frequent HNSCC alterations, aimed at developing effective targeted preventive strategies.

The biochemical and biological effects of metformin were evaluated in representative HNSCC cells expressing mutated *PIK3CA* or HPV oncogenes (HPV+). The oral delivery of metformin was optimized to achieve clinical relevant blood levels. Molecular determinants of metformin sensitivity were also investigated, and their expression levels examined in a large collection of HNSCC cases.

[*]Corresponding Author: J. Silvio Gutkind, Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, NIH, 30 Convent Drive, Building 30, Room 320, Bethesda, MD 20892. Phone: (301) 496-6259; Fax: (301) 402-0823; sg39v@nih.gov.

[4,5,6,7]HNC SPORE HIV ARRA Supplement Consortium.

Disclosure of Potential Conflicts of Interest: The authors indicate no disclosures of potential conflicts of interest.

We found that metformin inhibits mTOR signaling and tumor growth in HNSCC cells expressing mutated *PIK3CA* and HPV oncogenes, and that these activities require the expression of organic cation transporter 3 (OCT3/SLC22A3), a metformin uptake transporter. Co-expression of OCT3 and the mTOR pathway activation marker pS6 were observed in most HNSCC cases, including those arising in HIV+ patients.

Activation of the PI3K-mTOR pathway is a widespread event in HNSCC, including HPV− and HPV+ lesions arising in HIV+ patients, all of which co-express OCT3. These observations may provide a rationale for the clinical evaluation of metformin to halt HNSCC development from precancerous lesions, including in HIV+ individuals at risk of developing HPV-associated cancers.

Keywords

mTOR; cancer prevention; metformin; HIV; HPV

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is one of the six most common cancers in the world (1). Its traditional risk factors, including tobacco and alcohol consumption, have recently declined (2). However, there is an increase in the incidence of HNSCC associated with human papilloma virus (HPV) infection, which continues to rise worldwide (3, 4). Infection with high-risk HPV, mostly HPV16, is also associated with cervical and most anal cancers (4–6). This is of particular relevance for individuals that are positive for human immunodeficiency virus (HIV+), who are prone to develop HPV-related malignancies. For example, a study of HIV-infected patients found that 72% of them were co-infected with HPV (7). A large scale study of HIV+ and negative (HIV−) patients identified that the odds of developing anal, vaginal and oropharyngeal SCCs were increased 43, 21 and 3 times respectively, when compared to a control patient population (8). This and other studies indicate that HIV+ individuals that are also infected with HPV constitute a high risk group for SCC development (6, 9).

Most HNSCC and cervical SCC lesions exhibit strongly elevated activity of the PI3K and mammalian target of rapamycin (mTOR) signaling pathway (10–13). The activation of the mTOR pathway renders these cancers sensitive to the inhibition of mTOR by rapamycin and related rapalogs and new mTOR kinase inhibitors, as revealed in multiple pre-clinical animal models and experimental systems (14). While these data suggest that inhibition of the mTOR pathway with rapamycin or other signaling inhibitors may represent a suitable strategy for HNSCC treatment and/or prevention (15), prolonged use of mTOR inhibitors may have immunosuppressive activity (16), which is of special concern in HIV-infected individuals that are at risk of developing AIDS.

Of interest, recent findings suggest that metformin can efficiently inhibit the malignant progression of oral premalignant lesions in chemically-induced experimental models (17), and diminishes tumor growth in HNSCC xenografts (18, 19). Metformin is used as first-line oral drug for the treatment of type 2 diabetes mellitus, and is among the top 10 drugs prescribed in the United States, with more than 60 million prescriptions in 2012. Its use has

strongly increased in the last 5 years (20). Metformin is also used for the treatment of polycystic ovary syndrome (21) and, importantly, to manage lipodystrophy in HIV+ patients undergoing highly active anti-retroviral therapy (HAART) (22, 23). Here, we optimized the oral delivery of metformin to achieve clinically relevant blood levels, and explored its preclinical activity in representative HNSCC tumor xenograft models. We now show that metformin prevents the growth of malignant lesions arising from HNSCC cells harboring *PIK3CA* mutations or *HPV* oncogenes, and that these effects depend on the expression of organic cation transporter 3 (OCT3/SLC22A3), a metformin uptake transporter (24). Furthermore, activation of the PI3K-mTOR pathway was found to be widespread in head and neck, cervical, and anal cancer, including HPV− and HPV+ SCC lesions arising in HIV + patients. More importantly, these SCCs express high levels of OCT3, hence making these lesions potentially sensitive to metformin. These findings raise the possibility of exploring the clinical efficacy of metformin to prevent SCC development, including in HIV-infected individuals that are at high risk of developing HPV-associated cancers.

Materials and methods

Reagents, cell lines, tissue culture

Human-derived HNSCC cell lines CAL27 (ATCC), CAL33 and UMSCC47 were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Sigma Aldrich) at 37°C in humidified air with 5% CO_2. All cell lines underwent DNA authentication (Genetica DNA Laboratories, Inc.) prior to the described experiments to ensure consistency in cell identity. See Supplementary Materials for additional details.

Lentiviral constructs for OCT3 knock-down

Four shRNA constructs targeting human OCT3 were obtained from Open Biosystems. The 21 bp targeting sequences were: 5′-AAGAATAAGTGTGCATTTC-3′ (clone ID V2LHS_98079), 5′-TTTAGGGTGACAGTGGAGG-3′ (V2LHS_232514), 5′-TTGATGAGAGGTATTTCCC-3′ (V3LHS_371553), 5′-ATTTCTGTCACAATCACGT-3′ (V3LHS_371552). GIPZ Non-silencing Lentiviral shRNA Control was also obtained from Open Biosystems. See Supplementary Materials for additional details on virus production and transduction.

Western blotting, cell proliferation and colony formation

Antibodies were used from Cell Signaling Technology against ribosomal protein S6, phospho-S6 (pS6; Ser240/244), total AMPK, phospho-AMPKα (pAMPKα; Thr172), total 4E-BP, non-phospho-4E-BP (T46), total AKT1, phosphor-AKT1 (pAKT1; Ser476), and α-tubulin. Antibodies against OCT3 were obtained from Abcam. See Supplementary Materials for additional details.

For proliferation assays, cells were grown in 24-well plates and incubated with 0.5 μCi [^{3}H]-thymidine/mL (Perkin Elmer) for the last 4 hours of treatment, washed twice with ice cold PBS, and then thrice with cold 10% trichloroacetic acid for 10 minutes at 4°C. Cells were

then lysed in 0.5 ml 0.3N NaOH for 1 hour at 4°C. Samples (250 μl) were mixed with 5 ml of scintillation fluid and radioactivity counted in a liquid scintillation counter.

For colony forming assays, see Supplementary Materials.

Xenograft HNSCC tumor models

All animal studies were carried out according to NIH-approved protocols, in compliance with the Guide for the Care and Use of Laboratory Animals. Female 4- to 6-week-old nude mice (NCRNU-F, Taconic Farms) were injected subdermally in flanks with 1 million of CAL27, CAL33 or UMSCC47 cells. The day after injection they were given either water (control) or metformin in the drinking water at 2.5 mg/ml (or as indicated). See Supplementary Materials for additional details.

Histological studies and immunohistochemistry

Two tissue arrays containing 120 cases of oral cancer and 100 cases of cervical cancer were used for general evaluation and controls. For oral cancer in HIV+ patients we used a specific tissue microarray originally containing 229 cores of which a minimum of 128 were available for different stainings. We also used primary cases of HIV+ patients with HPV-positive perianal, cervical and oral tumors. Tissues from oral cancer HIV+ patients, including the tissue microarray, were available through the Head and Neck Cancer SPORE HIV Consortium. Unstained and H&E slides from oral, cervical, and perianal squamous cell carcinomas from HIV+ patients, were kindly provided by the AIDS and Cancer Specimen Resource, funded by the National Cancer Institute AIDS and Cancer Specimen Resource (ACSR, University of California, San Francisco and ACSR East Region, George Washington University). See Supplementary Materials for additional details.

Statistical analysis

ANOVA followed by the Tukey t-test were used to analyze the differences between groups after treatments. Data analysis was done with GraphPad Prism version 5.01 for Windows (GraphPad Software); p-values of less than 0.05 were considered statistically significant. Two-tailed, unpaired t-tests were used to analyze the differences in tumor growth between experimental groups.

Results

Metformin inhibits mTOR signaling in HNSCC cell lines *in vitro*

The mechanism of action of metformin is complex, and includes the mild inhibition of the mitochondrial complex I, leading to increased adenosine monophosphate (AMP) and lowered adenosine triphosphate levels (ATP). This results in the activation of AMP-activated protein kinase (AMPK) signaling (25), which in turn can reduce mTOR activity in its complex 1 (mTORC1) (26). Hence metformin represents an attractive drug candidate to prevent the development of cancer lesions that involved mTOR activity. These include HNSCCs, in which multiple molecular mechanisms converge on the stimulation of mTORC1 and the consequent accumulation of the phosphorylated form of ribosomal protein S6 (pS6) and other mTORC1 targets (10, 11, 27).

To begin addressing whether metformin inhibits the mTOR pathway in HNSCC cells harboring distinct genomic alterations, we chose three representative cell lines that carry a broad range of HNSCC-associated genetic alterations: CAL33 carrying an activating mutation in *PIK3CA* (H1047R (28)), UMSCC47 that is positive for HPV16 (29), and CAL27 carrying multiple potentially tumorigenic mutations in genes including *TP53* and *NOTCH2*, but without known mutations in the PI3K/AKT pathway (unpublished data). We found that when treated with metformin, all three cell lines exhibited a marked decrease of pS6, one of the major downstream targets of the mTOR pathway (Fig. 1A). Decreased pS6 (S240/244) was most prominent in UMSCC47, but could be observed in CAL27 and CAL33 at 1 mM and 3 mM metformin concentrations (Fig. 1B). Rapamycin was used as a control, which completely abrogated pS6 expression, indicating the presence of a functional mTOR pathway. Align with their known activities, treatment with metformin but not rapamycin resulted in increased pAMPK levels, while both treatments diminished pS6 (Fig. 1B). Thus, metformin downregulates the activity of mTOR pathway in HNSCC cell lines *in vitro*. Of interest, we did not observe consistent changes in AKT phosphorylation at S473, a target for mTORC2 (30), in response to metformin, which suggests that its effects on mTOR are specific for mTORC1.

Metformin inhibits HNSCC cell proliferation *in vitro*

We next investigated whether the effects of metformin on cell signaling would result in changes in cell proliferation. For that, we determined the rate of DNA-synthesis in cells by measuring [H^3]-thymidine incorporation. We found that metformin significantly reduced cell proliferation in CAL27, CAL33 and UMSCC47 cell lines (Fig. 1C). Again, the HPV+ UMSCC47 cell line exhibited the strongest sensitivity to metformin with a significant decrease in cell proliferation at lower metformin concentrations. We also found that metformin significantly decreased colony size in colony forming assay (Figs. 1D, S1). These data suggest that the changes in cell signaling caused by metformin affect the proliferative capacity of HNSCC cells.

Metformin inhibits HNSCC tumor growth *in vivo*

The *in vitro* effects of metformin on the HNSCC cells prompted us to study its effect on tumor progression *in vivo* (Fig. 2A). In order to achieve metformin concentration that would be relevant in the clinical setting, we first analyzed metformin concentrations in the plasma of mice after the oral administration of different doses of metformin. As shown in Figure 2B, 2.5 mg/ml of metformin in the drinking water resulted in approximately 2 μg/ml of metformin in plasma, which is within the concentration found in human patients treated with this drug for type 2 diabetes (Fig. 2B) (31, 32). We then transplanted CAL27, CAL33 and UMSCC47 cells into nude recipient mice, and randomly distributed the mice into control and metformin treated groups (Fig. 2A). We found a dramatic decrease of the tumor progression in all HNSCC xenografts, which was reflected by the total tumor burden at the time of sacrifice in all mice treated with metformin (Fig. 2C). Both size and weight of the tumors were lower for all three cell lines in the metformin-treated group when compared to the control groups. There was no difference in body weight of mice between the control and treated groups (data not shown).

Metformin inhibits mTOR pathway in xenograft tumor models

For further characterization of metformin effects and mechanisms of action, we focused on UMSCC47 cells, due to their relevance to the rising number of HPV+ cases of HNSCC. Tumor bearing mice were treated with control drinking water or metformin or with rapamycin as a control (see Materials and Methods), for 3 days. Analysis of the processed tumors revealed a strong reduction in pS6 levels upon treatment with metformin and rapamycin, whereas the non-phosphorylated fraction of 4E-BP1 protein, an important downstream target of the mTOR pathway, increased, indicating a cumulative decrease of 4E-BP1 phosphorylation (Fig. 3A). There were no consistent changes in pAKT levels, again suggesting that metformin affects mTORC1 *in vivo*. pS6 levels were also quantified after IHC staining of tumor sections, showing a significant decrease of pS6 in the tumors treated with metformin or rapamycin in comparison to the untreated control tumors, paralleling the results obtained by Western blotting (WB) (Fig. 3B, D). The tumor sections also showed a clear decrease in the fraction of proliferating cells that were detected as BrdU+ in the tumors treated with metformin or rapamycin (Fig. 3C, D). These results indicate that the oral administration of metformin inhibits mTOR pathway and decreases the proliferation of tumor cells *in vivo*.

OCT3 expression is essential for metformin effects on AMPK and mTOR signaling

Metformin is a very hydrophilic, membrane-impermeable compound that requires active transport to be incorporated into the cell (33). Therefore, its effects may depend on the expression and functional activity of organic cation transporters belonging to the *SLC22A* gene family (24, 34, 35). While its key metabolic effects in type 2 diabetic and polycystic ovary syndrome patients are likely dependent on the expression of OCT1 in the liver (34, 36), OCT1 is absent or minimally expressed in HNSCC cells (37). Instead, these cells express OCT3 (a widely expressed organic cation transporter), as judged by Western blotting in CAL27, CAL33 and UMSCC47 cells. All three cell lines exhibited presence of OCT3 expression (Fig. 4A).

To determine whether OCT3 expression is necessary for metformin function in HNSCC cells, we infected UMSCC47 cells with lentiviral particles expressing a pool of four shRNAs specific for human OCT3. Expression of the shRNAs resulted in more than 70% decrease of OCT3 in the cells (Fig. 4A). The impact of knocking down OCT3 (OCT3 KD) on metformin activity was then assessed, using cells infected with a non-specific shRNA lentivirus as control. We observed that pS6 was, as expected, significantly reduced in the control cells upon treatment with either metformin or rapamycin, but metformin had no visible effect on S6 phosphorylation in OCT3 KD cells. Moreover, there was no increase in AMPK phosphorylation in the OCT3 KD cells (Fig. 4B). The effect of pS6 reduction was further quantified. We did not observe any significant effect after treating OCT3 KD cells with metformin (Fig. 4C). Taken together, these data show that reduction of OCT3 expression level significantly reduced the ability of metformin to affect AMPK and mTOR signaling *in vitro*.

Reduction of OCT3 diminishes metformin effect on cell proliferation *in vitro* and its antitumoral effect *in vivo*

The reduction of metformin effect on mTOR signaling in OCT3 KD UMSCC47 cells prompted us to examine whether metformin would still affect cell proliferation of cells in which OCT3 expression is reduced. OCT3 KD cells displayed a clearly reduced response to the growth inhibitory effect of metformin, and although they still responded to higher metformin concentrations, control cells were more sensitive to metformin than OCT3 KD cells (Fig. 4D). Rapamycin treatment exhibited similar effect for both cell populations, thus serving as a specificity control. We observed similar results for colony forming assays. The size of the colonies of OCT3 KD cells did not change in response to metformin, at concentrations in which metformin significantly decreased cell colony growth in control cells. In all cases the effect of metformin on OCT3 KD was significantly weaker than its effect on the control cells at the same concentration, without altering the response to rapamycin (Fig. 4E).

Similarly, UMSCC47 OCT3 KD cells did not respond to metformin *in vivo*. Nude mice were implanted with UMSCC47 control cells or UMSCC47 OCT3 KD cells. The day after implantation half of the mice from each group started receiving metformin as described above. Only the mice that were implanted with control UMSCC47 cells and treated with metformin exhibited reduced tumor volume (Fig. 4F) and weight at the time of sacrifice (Fig. 4G). Tumor bearing mice were also treated for a short-term with either metformin or rapamycin as described above and tumors were analyzed by WB for mTOR pathway activity. We found a significant decrease of pS6 and an increase of pAMPK in the control tumors that were treated with metformin *in vivo*, while the control tumors treated with rapamycin had an even stronger reduction of pS6, but no significant increase in pAMPK levels (Fig. 4B). In contrast, we did not observe significant changes in pS6 and pAMPK levels in the metformin treated UMSCC47 OCT3 KD-derived tumors, whereas the changes caused by rapamycin were similar, indicating that the UMSCC47 OCT3 KD-derived tumors were less sensitive to metformin *in vivo* as well as *in vitro* (Fig. 4B). Taken together, these data support the important role of OCT3 expression in the response to metformin in tumor signaling and progression.

Expression of OCT3 in normal oral epithelium and in HPV− and HPV+ HNSCC lesions

To explore relevance of the data described above to human cancers, we evaluated the expression of pS6, p16 (a surrogate marker for HPV infection), and OCT3 in HNSCC tissues, using normal oral mucosal as a control. In normal oral mucosa (Fig. 5A, top), the activation of mTOR, as judged by pS6 expression, is compartmentalized, and limited to the upper cell layers, whereas basal and parabasal cell layers usually show no activation; p16 is not expressed, and the OCT3 transporter is present in all epithelial cells, including those of the proliferating basal and parabasal layers. In HPV− HNSCCs (Fig. 5A, middle), the mTOR pathway becomes deregulated and overactivated, and its downstream phosphorylation target pS6 accumulates in the cytoplasm of almost 90% of all tumor cells, with most cancer cells expressing OCT3 but not p16. In HPV+ HNSCCs (Fig. 5A, bottom) deregulation of mTOR is similar to that of HPV− tumors, but p16 is clearly expressed in tumor cells. Importantly, malignant cells also show strong deregulation of the mTOR

pathway (Fig. 5B), regardless of the HPV status (Fig. 5C), as well as OCT3 immunoreactivity, which is readily detectable in HPV+ HNSCC cases (Fig. 5D). Taken together, these data indicate that human HPV+ and HPV− HNSCC tissues exhibit expression of surrogate markers that suggest sensitivity to metformin, such as upregulated pS6 and OCT3 expression.

Expression of surrogate markers for sensitivity to metformin in HNSCC and cervical tumors arising in HIV+ patients

HIV+ patients have high risk of developing SCC. To determine expression of surrogate markers for metformin sensitivity, a series of oral and cervical cancer tissue microarrays, including a large number of histological cores from HIV+ patients, were evaluated for p16, pS6 and OCT3 expression by immunohistochemistry. We also included examples of primary cases of oral, uterine cervix, and perianal SCC lesions from HIV-infected patients. There was a significant increase in the fraction of HNSCC lesions positive for the HPV-infection surrogate p16 (35%) in HIV+ patients, when compared to HIV− patients (21%) (Fig. 6A, B). Regardless of their HIV or HPV status, the majority of the tumor cells express pS6 (Fig. 6A, C) and the OCT3 transporter (Fig. 6A, D). Representative cores of the arrays are shown at different magnifications (Fig. 6A). The lower two rows show the immunoreactivity of representative extraoral tumors, cervical and anal, and their morphological features by H&E staining. These lesions are also positive for pS6 and OCT3 (Fig. 6E). An example depicting the expression of p16, pS6 and OCT3 in the proliferative compartment of an anal HPV+ SCC lesion from an HIV+ patient is also shown in supplemental figure 2. Thus, deregulation of pS6 and co-expression of OCT3 are not only characteristic for HNSCC, but are observed in other SCC lesions, including cervical and anal cancers arising in HIV+ patients.

Discussion

In this study, we show that metformin reduces the proliferation *in vitro* of HNSCC cells harboring mutations in *PIK3CA* or derived from HPV+ HNSCC lesions, both of which are frequent events in oral malignancies. Furthermore, by optimizing the oral delivery of metformin to achieve clinically relevant blood levels, we now show that metformin is highly effective in reducing HNSCC tumor growth *in vivo.* Metformin activated AMPK and decreased mTOR activity in HNSCC *in vitro* and *in vivo*. Importantly, these effects required the expression of the organic cation transporter OCT3, a well-known uptake transporter of small hydrophilic organic cationic endogenous compounds, as well as toxins and drugs, including metformin (38). These findings support the emerging notion that metformin may act on the cancer and pre-cancer cells directly, rather than exerting a cancer preventive activity solely based on its systemic metabolic effects. Furthermore, the immunodetection of mTOR hyperactivity in HNSCC and high OCT3 expression levels in normal oral epithelium and all analyzed HNSCC cases provide surrogate markers that may predict a favorable response to metformin in HNSCC and other SCC lesions. In particular, this information raises the possibility of using metformin to prevent the progression of squamous cancers in at risk patient populations, including the increasing incidence of HPV-associated oral, cervical, and anal SCC arising in HIV-infected individuals.

The use of HAART has dramatically reduced the incidence of acquired immune deficiency syndrome (AIDS), and consequently the number of patients affected by some AIDS-defining malignancies, such as Kaposi's sarcoma and Non-Hodgkin lymphoma (8). However, HAART showed limited success in diminishing the incidence of non-AIDS-defining cancers, including oral and anal cancer, which instead have been steadily increasing as the population of HIV-infected individuals increase and age (8, 39). Hence, there is an urgent need to develop suitable strategies to prevent the development of HPV-associated SCCs in the general population and in at risk groups, including HIV+ patients. In this regard, metformin is considered safe, although in very rare circumstances, such as in renal deficiency, it can cause lactic acidosis (33). Besides its use in type II diabetes, metformin is also used for treatment lypodistrophy in HIV-infected patients undergoing HAART (21, 22, 34). Hence, there is a considerable clinical experience with the beneficial and potential side effects of metformin in HIV-infected individuals, which can now be considered when assessing its potential use for cancer prevention in this at risk patient population.

The precise mechanism by which metformin acts in HNSCC is not fully understood. In principle, metformin limits mTOR activity, primarily modulating mTORC1 complex as judged by the reduction of phosphorylation of its downstream effectors, 4E-BP1 and S6 (17, 40). Instead, phosphorylation of AKT at S473 residue, a typical target for mTORC2 (30), was not affected. We also observed activation of AMPK resulting from increased AMPK phosphorylation in its activating residue. These observations suggest that mTOR regulation might result from a described pathway involving the activation of AMPK that leads to an increased TSC2 GTPase activity upstream of mTORC1 (41), a step acting downstream of PI3K, therefore explaining sensitivity to metformin of the *PIK3CA*-mutated CAL33 cell line. Enhanced AMPK activity is in turn expected to occur following the inhibition of mitochondrial complex I by metformin. However, metformin may also act through other potential AMPK-independent mechanisms involving Rag GTPase (42) and the mTOR inhibitor REDD1 (43). These mechanisms are not mutually exclusive and their relative importance for the effect of metformin could be tumor-specific, which requires further investigation.

Another important issue to consider is the possibility that metformin acts by reducing the circulating levels of insulin and insulin-like growth factor (IGF-1) (44). While this effect is likely of importance for the beneficial effect of metformin in patients suffering conditions that exhibit high levels of insulin, such as in type 2 diabetes, polycystic ovary syndrome or insulin-dependent malignancies, we have recently shown that metformin reduces the incidence of conversion of premalignant lesions into oral SCCs in a non-diabetic chemically-induced oral cancer model (17). Furthermore, we obtained evidence that metformin reduces the activity of mTOR in basal epithelial cells within dysplastic lesions (17), which may represent the population of origin of oral SCCs. Indeed, we now provide direct evidence that metformin reduces the activity of mTOR in HNSCC cells. In support of these recent findings, we now observed that reducing OCT3 levels in HNSCC cells prevents the growth suppressive consequences of metformin administration. Moreover, OCT3 knock down inhibited the biochemical activation of AMPK and mTOR suppression in response to

metformin. These data lend further support to the concept that metformin may act directly on the cancer cells *in vivo*, instead of, or in addition to, its other systemic metabolic effects.

Unlike phenformin, metformin requires an active uptake mechanism into its target cells. One of the transporters that can deliver metformin into cells is the highly expressed in liver OCT1, therefore making hepatic cancer a prominent target for metformin. Other cancers may be resistant to metformin due to lack of necessary organic cation transporters (35) or by an active efflux of metformin mediated by transporters involved in multidrug resistance (45, 46). In principle, we can envision that metformin will be effective in cancers exhibiting activated mTOR pathway, provided that the cancer cells or their potential premalignant lesions also express OCT3 or other transporters favoring metformin intracellular accumulation instead of its efflux. In this regard, multiple observational and meta-analysis studies indicate that patients taking metformin have lower incidence of several malignancies in comparison to relevant control populations, including cancers of the breast, liver, lung, colon, pancreas and prostate (reviewed in (47, 48). Furthermore, recent studies also suggest that the use of metformin can specifically reduce the incidence of head and neck cancer in diabetics (49). Whether the potential beneficial activity of metformin in these multiple cancer types is dependent on OCT3 expression or other metformin transporters can now be examined.

Specifically for HNSCC, surgery, radiation, and chemotherapy, as well as targeted agents such as cetuximab, are the current standard treatment, while new agents targeting PIK3CA, mTOR, and the immune response are now under clinical evaluation in multiple trials (50). However, it is unlikely that any of these therapeutic options will be suitable for prophylactic use due to often severe side effects and/or high cost. In addition, HNSCC is a highly recurring cancer, with one-fifth of the patients diagnosed with secondary malignancies within 5 years after a successful surgery and/or radiation therapy (1). This fact makes cancer-free HNSCC survivors another high risk group that currently cannot undergo any preventive therapy to decrease the probability of a second cancer, except for re-irradiation, which is used with mixed results (51). Therefore, metformin could be potentially used as a preventive strategy for HNSCC and other SCC cancer survivor patients after definitive treatment. Overall, we can conclude that prior studies and our present findings support the early evaluation of metformin as a relatively safe and low cost preventive strategy for the prophylaxis of cancer development in patients at risk of HNSCC and multiple HPV-associated malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: D. Madera, L. Vitale-Cross, D. Martin, A.A. Molinolo and J.S. Gutkind were supported by the Intramural Program of the National Institute of Dental and Craniofacial Research, National Institutes of Health. T.E. Carey, J.B. McHugh, C.M. Komarck, H.M. Walline, W.N. William, R.R. Seethala and R. Ferris were supported by the Head and Neck SPORE Consortium NCI supplement # P50 CA097248.

This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Dental and Craniofacial Research, and by the Head and Neck SPORE Consortium NCI supplement. We thank the AIDS and Cancer Specimen Resource, funded by the National Cancer Institute AIDS and Cancer Specimen Resource (ACSR, University of California, San Francisco and ACSR East Region, George Washington University) for providing oral, cervical, and perianal squamous cell carcinomas from HIV+ patients.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013; 63:11–30. [PubMed: 23335087]
2. Warnakulasuriya S. Causes of oral cancer - an appraisal of controversies. Br Dent J. 2009; 207:471–5. [PubMed: 19946320]
3. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2011; 29:4294–301. [PubMed: 21969503]
4. Jemal A, Simard EP, Dorell C, Noone A-M, Markowitz LE, Kohler B, et al. Annual Report to the Nation on the Status of Cancer, 1975–2009, Featuring the Burden and Trends in Human Papillomavirus (HPV)–Associated Cancers and HPV Vaccination Coverage Levels. J Natl Cancer Inst. 2013
5. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. Journal of clinical pathology. 2002; 55:244–65. [PubMed: 11919208]
6. Chaturvedi AK, Madeleine MM, Biggar RJ, Engels EA. Risk of human papillomavirus-associated cancers among persons with AIDS. J Natl Cancer Inst. 2009; 101:1120–30. [PubMed: 19648510]
7. Tamalet C, Obry-Roguet V, Ressiot E, Bregigeon S, Del Grande J, Poizot-Martin I. Distribution of human papillomavirus genotypes, assessment of HPV 16 and 18 viral load and anal related lesions in HIV positive patients: A cross-sectional analysis. J Med Virol. 2014; 86:419–25. [PubMed: 24154930]
8. Patel P, Hanson DL, Sullivan PS, Novak RM, Moorman AC, Tong TC, et al. Incidence of Types of Cancer among HIV-Infected Persons Compared with the General Population in the United States, 1992–2003. Ann Intern Med. 2008; 148:728–36. [PubMed: 18490686]
9. Blitz S, Baxter J, Raboud J, Walmsley S, Rachlis A, Smaill F, et al. Evaluation of HIV and Highly Active Antiretroviral Therapy on the Natural History of Human Papillomavirus Infection and Cervical Cytopathologic Findings in HIV-Positive and High-Risk HIV-Negative Women. J Infect Dis. 2013; 208:454–62. [PubMed: 23624362]
10. Coppock JD, Wieking BG, Molinolo AA, Gutkind JS, Miskimins WK, Lee JH. Improved clearance during treatment of HPV-positive head and neck cancer through mTOR inhibition. Neoplasia. 2013; 15:620–30. [PubMed: 23730210]
11. Molinolo AA, Marsh C, El Dinali M, Gangane N, Jennison K, Hewitt S, et al. mTOR as a Molecular Target in HPV-Associated Oral and Cervical Squamous Carcinomas. Clin Cancer Res. 2012; 18:2558–68. [PubMed: 22409888]
12. Amornphimoltham P, Patel V, Sodhi A, Nikitakis NG, Sauk JJ, Sausville EA, et al. Mammalian Target of Rapamycin, a Molecular Target in Squamous Cell Carcinomas of the Head and Neck. Cancer Res. 2005; 65:9953–61. [PubMed: 16267020]
13. Feng W, Duan X, Liu J, Xiao J, Brown RE. Morphoproteomic evidence of constitutively activated and overexpressed mTOR pathway in cervical squamous carcinoma and high grade squamous intraepithelial lesions. International journal of clinical and experimental pathology. 2009; 2:249–60. [PubMed: 19079619]
14. Nguyen AS, Walker D, Gillespie MB, Gutkind JS, Day AT. mTOR Inhibitors and its Role in the Treatment of Head and Neck Squamous Cell Carcinoma. Curr Treat Options Oncol. 2012; 13:71–81. [PubMed: 22282394]
15. Vander Broek R, Snow GE, Chen Z, Van Waes C. Chemoprevention of head and neck squamous cell carcinoma through inhibition of NF-kappaB signaling. Oral oncology. 2014; 50:930–41. [PubMed: 24177052]

16. Kaplan B, Qazi Y, Wellen JR. Strategies for the management of adverse events associated with mTOR inhibitors. Transplant Rev (Orlando). 2014 Epub ahead of print.
17. Vitale-Cross L, Molinolo AA, Martin D, Younis RH, Maruyama T, Patel V, et al. Metformin Prevents the Development of Oral Squamous Cell Carcinomas from Carcinogen-Induced Premalignant Lesions. Cancer Prev Res (Phila). 2012; 5:562–73. [PubMed: 22467081]
18. Luo Q, Hu D, Hu S, Yan M, Sun Z, Chen F. In vitro and in vivo anti-tumor effect of metformin as a novel therapeutic agent in human oral squamous cell carcinoma. BMC cancer. 2012; 12:517. [PubMed: 23151022]
19. Sikka A, Kaur M, Agarwal C, Deep G, Agarwal R. Metformin suppresses growth of human head and neck squamous cell carcinoma via global inhibition of protein translation. Cell Cycle. 2012; 11:1374–82. [PubMed: 22421144]
20. Hampp C, Borders-Hemphill V, Moeny DG, Wysowski DK. Use of Antidiabetic Drugs in the U.S., 2003–2012. Diabetes Care. 2014; 37:1367–74. [PubMed: 24623020]
21. Xiao J, Chen S, Zhang C, Chang S. The effectiveness of metformin ovulation induction treatment in patients with PCOS: a systematic review and meta-analysis. Gynecological endocrinology: the official journal of the International Society of Gynecological Endocrinology. 2012; 28:956–60. [PubMed: 22808990]
22. Hadigan C, Corcoran C, Basgoz N, Davis B, Sax P, Grinspoon S. Metformin in the treatment of hiv lipodystrophy syndrome: A randomized controlled trial. JAMA. 2000; 284:472–7. [PubMed: 10904511]
23. Sheth SH, Larson RJ. The efficacy and safety of insulin-sensitizing drugs in HIV-associated lipodystrophy syndrome: a meta-analysis of randomized trials. BMC infectious diseases. 2010; 10:183. [PubMed: 20573187]
24. Chen L, Pawlikowski B, Schlessinger A, More SS, Stryke D, Johns SJ, et al. Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. Pharmacogenetics and genomics. 2010; 20:687–99. [PubMed: 20859243]
25. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001; 108:1167–74. [PubMed: 11602624]
26. Shaw RJ. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. Acta Physiol (Oxf). 2009; 196:65–80. [PubMed: 19245654]
27. Czerninski R, Amornphimoltham P, Patel V, Molinolo AA, Gutkind JS. Targeting mammalian target of rapamycin by rapamycin prevents tumor progression in an oral-specific chemical carcinogenesis model. Cancer Prev Res (Phila). 2009; 2:27–36. [PubMed: 19139015]
28. Rebucci M, Peixoto P, Dewitte A, Wattez N, De Nuncques MA, Rezvoy N, et al. Mechanisms underlying resistance to cetuximab in the HNSCC cell line: role of AKT inhibition in bypassing this resistance. International journal of oncology. 2011; 38:189–200. [PubMed: 21109940]
29. Tang AL, Owen JH, Hauff SJ, Park JJ, Papagerakis S, Bradford CR, et al. Head and Neck Cancer Stem Cells: The Effect of HPV—An In Vitro and Mouse Study. Otolaryngology -- Head and Neck Surgery. 2013; 149:252–60. [PubMed: 23585151]
30. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. Science. 2005; 307:1098–101. [PubMed: 15718470]
31. Balan G, Timmins P, Greene DS, Marathe PH. In vitro-in vivo correlation (IVIVC) models for metformin after administration of modified-release (MR) oral dosage forms to healthy human volunteers. J Pharm Sci. 2001; 90:1176–85. [PubMed: 11536222]
32. Sambol NC, Chiang J, O'Conner M, Liu CY, Lin ET, Goodman AM, et al. Pharmacokinetics and pharmacodynamics of metformin in healthy subjects and patients with noninsulin-dependent diabetes mellitus. Journal of clinical pharmacology. 1996; 36:1012–21. [PubMed: 8973990]
33. Graham G, Punt J, Arora M, Day R, Doogue M, Duong J, et al. Clinical Pharmacokinetics of Metformin. Clin Pharmacokinet. 2011; 50:81–98. [PubMed: 21241070]
34. Gambineri A, Tomassoni F, Gasparini DI, Di Rocco A, Mantovani V, Pagotto U, et al. Organic cation transporter 1 polymorphisms predict the metabolic response to metformin in women with the polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2010; 95:E204–8. [PubMed: 20660041]

35. Yang J, Kalogerou M, Gallacher J, Sampson JR, Shen MH. Renal tumours in a Tsc1+/− mouse model show epigenetic suppression of organic cation transporters Slc22a1, Slc22a2 and Slc22a3, and do not respond to metformin. Eur J Immunol.
36. Christensen MM, Brasch-Andersen C, Green H, Nielsen F, Damkier P, Beck-Nielsen H, et al. The pharmacogenetics of metformin and its impact on plasma metformin steady-state levels and glycosylated hemoglobin A1c. Pharmacogenetics and genomics. 2011; 21:837–50. [PubMed: 21989078]
37. Patel H, Younis RH, Ord RA, Basile JR, Schneider A. Differential expression of organic cation transporter OCT-3 in oral premalignant and malignant lesions: potential implications in the antineoplastic effects of metformin. J Oral Path Med. 2013; 42:250–6. [PubMed: 22861817]
38. Nies, A.; Koepsell, H.; Damme, K.; Schwab, M. Organic Cation Transporters (OCTs, MATEs), In Vitro and In Vivo Evidence for the Importance in Drug Therapy. In: Fromm, MF.; Kim, RB., editors. Drug Transporters. Springer; Berlin Heidelberg: 2011. p. 105-67.
39. Palefsky JM, Holly EA, Efirdc JT, Da Costa M, Jay N, Berry JM, et al. Anal intraepithelial neoplasia in the highly active antiretroviral therapy era among HIV-positive men who have sex with men. Aids. 2005; 19:1407–14. [PubMed: 16103772]
40. Sinnett-Smith J, Kisfalvi K, Kui R, Rozengurt E. Metformin inhibition of mTORC1 activation, DNA synthesis and proliferation in pancreatic cancer cells: dependence on glucose concentration and role of AMPK. Biochem Biophys Res Commun. 2013; 430:352–7. [PubMed: 23159620]
41. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes & development. 2003; 17:1829–34. [PubMed: 12869586]
42. Kalender A, Selvaraj A, Kim SY, Gulati P, Brûlé S, Viollet B, et al. Metformin, Independent of AMPK, Inhibits mTORC1 in a Rag GTPase-Dependent Manner. Cell Metabolism. 2010; 11:390–401. [PubMed: 20444419]
43. Ben Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P, et al. Metformin, Independent of AMPK, Induces mTOR Inhibition and Cell-Cycle Arrest through REDD1. Cancer Res. 2011; 71:4366–72. [PubMed: 21540236]
44. Pollak M. Potential applications for biguanides in oncology. J Clin Invest. 2013; 123:3693–700. [PubMed: 23999444]
45. Hemauer SJ, Patrikeeva SL, Nanovskaya TN, Hankins GDV, Ahmed MS. Role of human placental apical membrane transporters in the efflux of glyburide, rosiglitazone, and metformin. American Journal of Obstetrics and Gynecology. 2010; 202:383.e1–e7. [PubMed: 20350646]
46. Choi CH. ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. Cancer cell international. 2005; 5:30. [PubMed: 16202168]
47. Miranda VC, Barroso-Sousa R, Glasberg J, Riechelmann RP. Exploring the role of metformin in anticancer treatments: a systematic review. Drugs of today. 2014; 50:623–40. [PubMed: 25313369]
48. Kasznicki J, Sliwinska A, Drzewoski J. Metformin in cancer prevention and therapy. Annals of Translational Medicine. 2014; 2:57. [PubMed: 25333032]
49. Yen YC, Lin C, Lin SW, Lin YS, Weng SF. Effect of metformin on the incidence of head and neck cancer in diabetics. Head Neck. 2014
50. Du Y, Peyser ND, Grandis JR. Integration of molecular targeted therapy with radiation in head and neck cancer. Pharmacol Ther. 2014; 142:88–98. [PubMed: 24280066]
51. Strojan P, Corry J, Eisbruch A, Vermorken JB, Mendenhall WM, Lee AWM, et al. Recurrent and second primary squamous cell carcinoma of the head and neck: When and how to reirradiate. Head & Neck. 2013 Epub ahead of print.

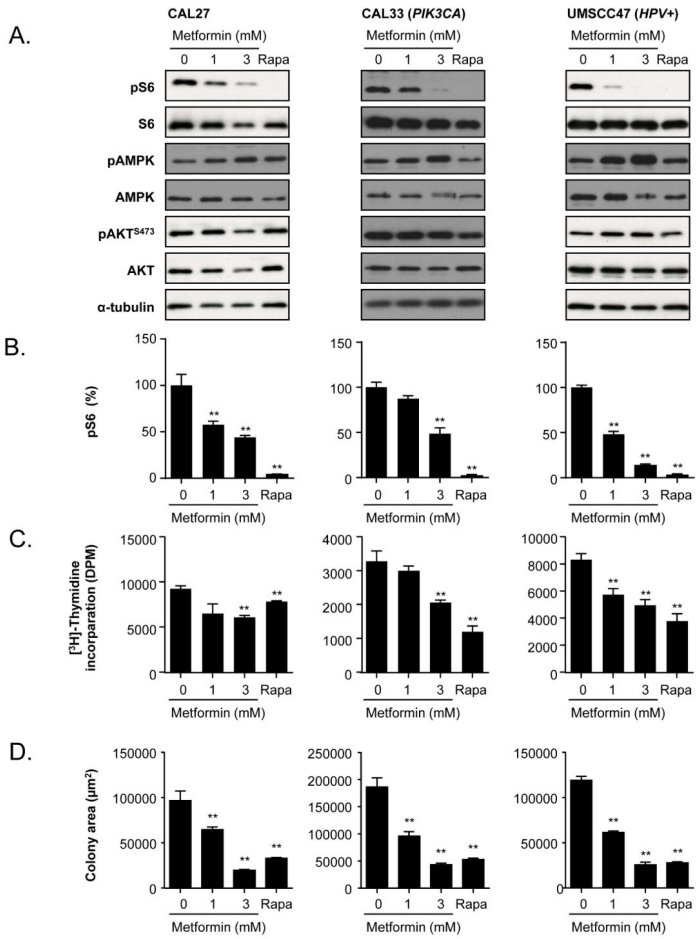

Figure 1.

(A) HNSCC cell lines CAL27, CAL33 and UMSCC47 were treated with the indicated concentrations of metformin or rapamycin, lysed and analyzed by WB for expression of pS6/S6, pAMPK/AMPK, pAKT/AKT, and α-tubulin as a loading control. (B) Cells were treated as described in (B) in triplicate for each condition. pS6 levels were analyzed by WB, quantified and normalized to S6. Values are the ratio of the means to the control ± SEM. (C) [^{3}H]-Thymidine incorporation assay of CAL27, CAL33 and UMSCC47 cells treated with metformin or rapamycin. (D) Colony formation assay of the HNSCC cell lines treated with metformin or rapamycin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

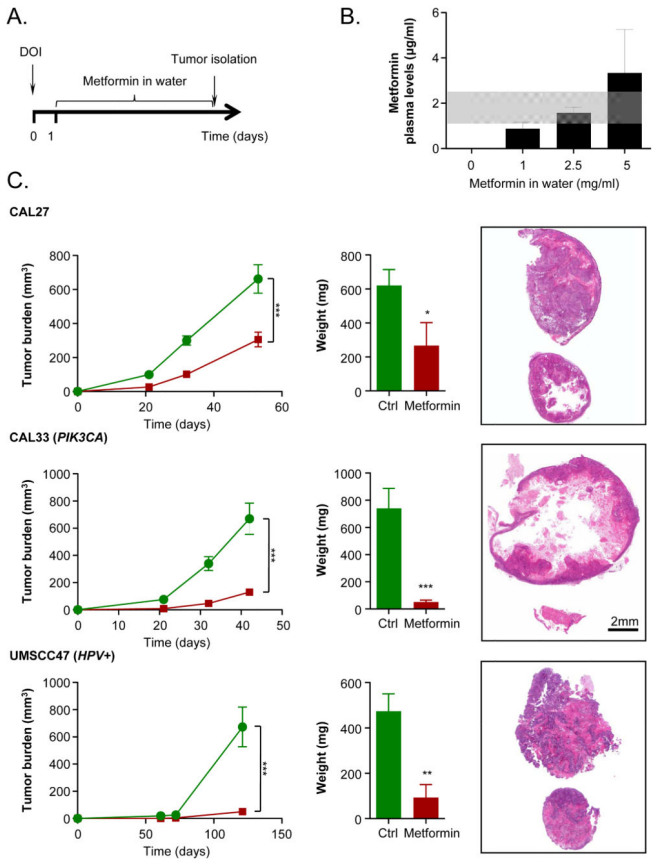

Figure 2.

(A) Schematic representation of time scale for the metformin treatment of mouse HNSCC xenograft models. (B) Metformin was delivered to mice through drinking water at the indicated concentrations for four days. Plasma from mouse blood was isolated, and metformin plasma concentration was measured. Semi-transparent gray area indicates typical range of metformin concentrations in plasma of diabetic patients and healthy control individuals treated with metformin. (C) Injected cells are indicated at the top left corner of each panel. Left: Time course of tumor growth with tumor volumes measured over time. Control tumor growth measurements are shown in green circles, and tumor growth measurements on metformin-treated mice are shown in red squares. Middle: Average weight of tumors at the end point of the experiment. Right: Hematoxylin and eosine (H&E) staining of tumor sections with control tumors shown on top and the tumors from metformin-treated mice shown at the bottom.

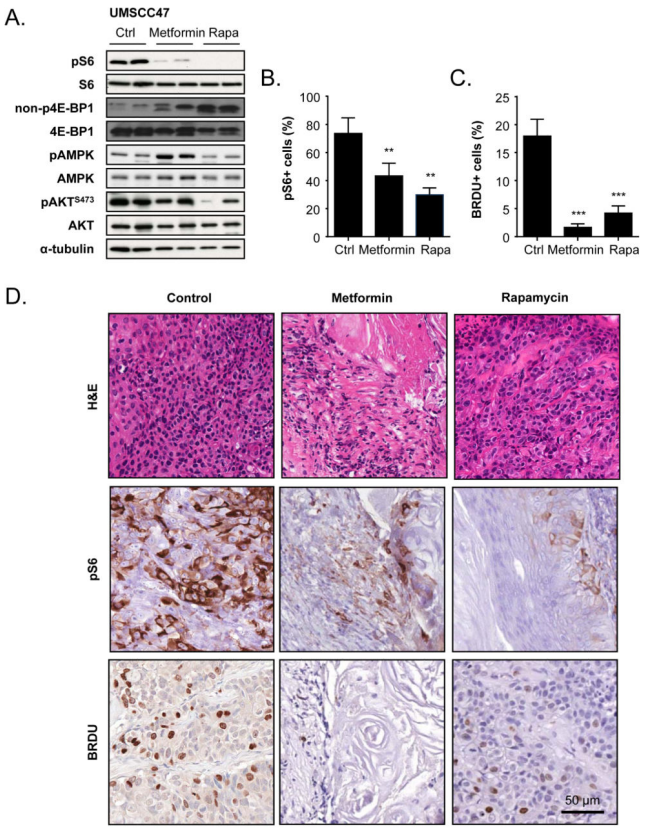

Figure 3.

UMSCC47 cells were injected into immunedeficient mice as described in Figure 2. When the mice developed palpable tumors, they were divided into three groups (n = 6 tumors for each group). The control group did not receive any treatment, while other groups were treated for three days with metformin in the drinking water (2.5 mg/ml) or with rapamycin at 5 mg/kg/day by intraperitoneal (i.p.) injections. (A) WB analysis of changes in mTOR signaling pathway, showed here in two separate tumors per treatment group. (B, C) Quantification of percentage of pS6+ (B) and BrdU+ (C) cells from the stained sections shown in (D). (D) Tumor stainings with H&E and immunohistochemistry (IHC) for pS6 and BrdU.

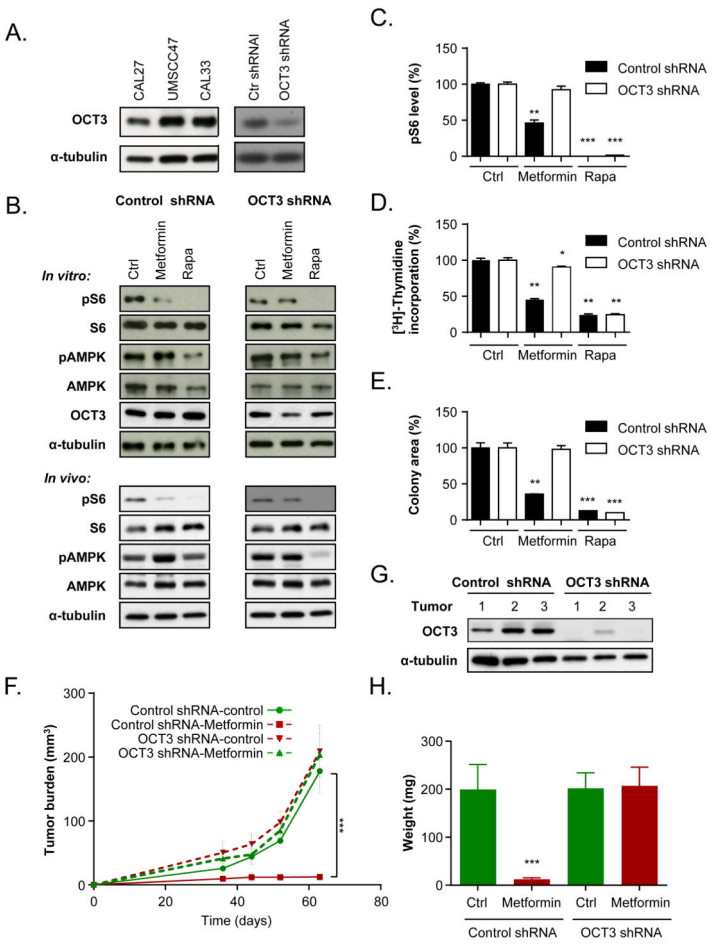

Figure 4.

UMSCC47 cells were infected with lentiviruses expressing a pool of shRNAs targeting human organic cation transporter (OCT3/SLC22A3) or non-specific control shRNAs. The UMSCC47-derived control and OCT3 shRNA cells were analyzed side by side for metformin effects on the cells. (A) WB analysis of OCT3 expression in the HNSCC cell lines (left) and UMSCC47-derived control and OCT3 shRNA cells (right). (B) Top: UMSCC47-derived cells were treated with metformin or rapamycin as described in Figure 1A, and expression of pS6/S6, pAMPK/AMPK was analyzed by WB. Metformin was used at 1 mM concentration, α-tubulin was used as a loading control. Bottom: Mice bearing UMSCC47-derived control or OCT3 shRNA tumors, were treated for three days with metformin or rapamycin as described in Figure 3. The tumors were isolated and analyzed by WB for pS6, S6, pAMPK, AMPK and α-tubulin expression. (C) Quantification of pS6 expression levels normalized to S6 with level in control set as 100% (n = 3). (D) [^{3}H]-Thymidine incorporation experiment was performed as described in Figure 1C with metformin at 1 mM concentration. The data was normalized to control values set as 100% (n = 4). (E) Colony formation assay was performed as described in Figure 1D with metformin at 1 mM concentration. The data was normalized to control values set as 100% (n = 4). (F) Time course of tumor growth with tumor volumes measured over time. Control UMSCC47 tumors shown in continuous lines, UMSCC47-OCT3 shRNA tumors shown in dotted lines.

Untreated tumors are depicted as green lines, metformin-treated tumors are depicted as red lines. (G) Expression of OCT3 in a panel of tumors described in Figure 4B. Three independent tumors derived from UMSCC47 control shRNA and OCT3 shRNA were used for the Western blot analysis, using α-tubulin as a loading control. (H) Average weight of tumors at the end point of the experiment.

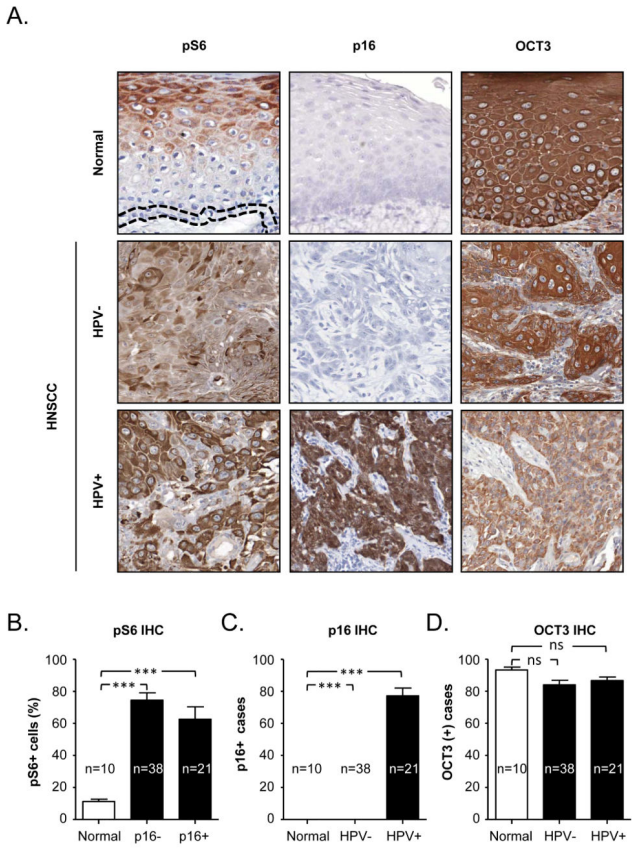

Figure 5.

A panel of human HNSCC patient samples was analyzed for expression of pS6 and OCT3 as biomarkers indicating mTOR signaling activation and potential sensitivity to metformin, respectively. (A) IHC staining of normal epithelium and HPV− and HPV+ HNSCC tumor sections for pS6, p16 and OCT3. (B, C, D) Quantification of the IHC staining results shown in figure 5A: (B) the percentage of pS6+ cells in normal tissues and in p16− and p16+ HNSCC tumors; (C) the percentage of p16+ cases in normal tissues, HPV− and HPV+ HNSCC tumors; and (D) the percentage of OCT3+ cases in normal tissues and HPV− or HPV+ HNSCC tumors. The number of tissues analyzed is indicated in each case.

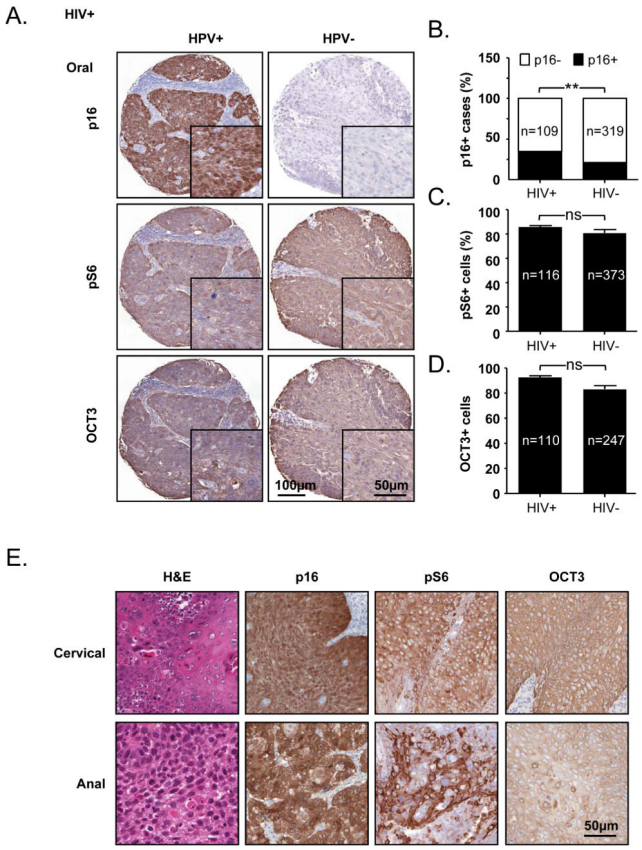

Figure 6.

A series of oral and cervical cancer tissue microarrays including histological cores from lesions arising in HIV+ patients were evaluated for p16 (HPV surrogate marker), pS6 and OCT3 expression by immunohistochemistry. In these studies we also included representative primary cases of HPV+ oral, uterine cervix and perianal SCC lesions from HIV+ patients. (A) IHC staining of HIV+ oral SCC showing p16, pS6 and OCT3 IHC stainings. Staining of a representative HPV+ sample is shown on the left, and HPV− on the right. (B) Quantification of the percentage of p16 positive cases in HIV+ and HIV− HNSCC tissues. (C) Quantification of the % of pS6+ cells in HIV+ and HIV− HNSCC tissues. (D) Quantification of the % of OCT3+ cells in HIV+ and HIV− HNSCC tissues. The number of tissues evaluated in each case is indicated. **, $p<0.01$. NS: no statistically significant difference. (E) Representative staining of cervical and anal SCC with H&E and IHC for p16, pS6 and OCT3.