Prevention of Tumor Growth Driven by \(PIK3CA\) and HPV Oncogenes by Targeting mTOR Signaling with Metformin in Oral Squamous Carcinomas Expressing OCT3

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Abstract

Most squamous cell carcinomas of the head and neck (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 biologic 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 were examined in a large collection of HNSCC cases. 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. Coexpression 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 coexpress 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. Cancer Prev Res; 8(3); 197–207.

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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 papillomavirus (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 coinfected with HPV (7). A large-scale study of HPV+ 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 with 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 rapalogues and new mTOR kinase inhibitors.
as revealed in multiple preclinical animal models and experimental systems (14). Although 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 II 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 poly cystic ovary syndrome (21) and, importantly, to manage lipodystrophy in HIV+ patients undergoing highly active antiretroviral therapy (HAART; refs. 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, thereby 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, and tissue culture

Human-derived HNSCC cell lines CAL27 (ATCC), CAL33, and UMSCC47 were grown and maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (Sigma-Aldrich) at 37°C in humidified air with 5% CO2. All cell lines underwent DNA authenticity 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 (ATP) levels. 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; ref. 26). Hence, metformin represents an attractive drug candidate to prevent the development of cancer lesions that involved mTOR activity. These include HNSCCs, in which

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Decreased pS6 (S240/244) was most prominent in UMSCC47, major downstream targets of the mTOR pathway (Fig. 1A). three cell lines exhibited a marked decrease of pS6, one of the but without known mutations in the PI3K

\[ \text{PIK3CA} \]

we chose three representative cell lines that carry a broad range of pathway in HNSCC cells harboring distinct genomic alterations, targets (10, 11, 27).

mTORC1 and the consequent accumulation of the phosphory-

ation mutation in

\( \text{PIK3CA} \)

regulates 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 \([H^\text{T}]\)-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 and Supplementary Fig. 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). 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 Fig. 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 II diabetes (Fig. 2B; refs. 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 with 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 nonphosphorylated 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

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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 with the untreated control tumors, paralleling the results obtained by Western blotting (Fig. 3B and D). The tumor sections also showed a clear decrease in the fraction of proliferating cells that were detected as bromodeoxyuridine-positive (BrdUrd+) in the tumors treated with metformin or rapamycin (Fig. 3C and 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). Although its key metabolic effects in type II 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 nonspecific 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, UMSSC47 OCT3 KD cells did not respond to metformin in vivo. Nude mice were implanted with UMSSC47 control cells or UMSSC47 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 UMSSC47 cells and treated with metformin exhibited reduced tumor volume (Fig. 4F). The data were normalized to control values set as 100% (n = 3). D, [3H]-thymidine incorporation experiment was performed as described in Fig. 1C with metformin at 1 mmol/L concentration. The data were normalized to control values set as 100% (n = 4). E, colony formation assay was performed as described in Fig. 1D with metformin at 1 mmol/L concentration. The data were normalized to control values set as 100% (n = 4). F, time course of tumor growth with tumor volumes measured over time. Control UMSSC47 tumors shown in continuous lines, UMSSC47- OCT3 shRNA tumors shown in dotted lines. Untreated tumors are depicted as green lines and metformin-treated tumors are depicted as red lines. G, expression of OCT3 in a panel of tumors described in B. Three independent tumors derived from UMSSC47 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 endpoint of the experiment.* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.
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 mucosa 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, bottom), mTOR pathway deregulation 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 TMAs, including a large number of histologic cores from lesions arising in HIV+ patients, were evaluated for p16 (HPV surrogate marker), pS6, and OCT3 expression by IHC. 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+ cases in HIV+ and HIV− HNSCC tissues. C, quantification of the percentage of pS6+ cells in HIV+ and HIV− HNSCC tissues. D, quantification of the percentage 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. **, P < 0.01; ns, not significant.
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 (IGF1; ref. 44). Although 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 II 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 nondiabetic 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 knockdown 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 actions.

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, not fully understood. In principle, metformin limits mTOR 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 population samples, 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 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 lymphodystrophy 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 (38), 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...
HSNCC survivors another high-risk group that currently cannot undergo any preventive therapy to decrease the probability of a second cancer, except for reirradiation, 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.

Disclosure of Potential Conflicts of Interest
J.S. Gutkind has ownership interest (including patents) in NIH, patent application for use of mTOR inhibitors for oral cancer prevention. No potential conflicts of interest were disclosed by the other authors.

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