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Anti-tumor effects of metformin on head and neck carcinoma cell lines: A systematic review

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Abstract. Metformin is commonly used for treating type 2 diabetes, and may also reduce cancer risk. Previous studies have demonstrated the association between metformin use and a decreased risk of head and neck cancer. Therefore, the aim of the present systematic review was to summarize the available literature on the *in vitro* anti-tumor effects of metformin on head and neck squamous cell carcinoma (HNSCC). Research studies were obtained from Cochrane Library, Embase, LILACS, MEDLINE and PubMed databases, without time or language restrictions. Only *in vitro* studies analyzing the effects of metformin on HNSCC cell lines were included. The authors methodically appraised all the selected studies according to the Grading of Recommendations Assessment, Development and Evaluation method to make a judgment of the evidence quality. Of the 388 identified reports, 11 studies met the inclusion criteria and were used for qualitative analysis. These studies demonstrated that metformin is important in inhibiting cell proliferation, inducing G0/G1 cell cycle arrest and apoptosis, and in regulating proteins involved in carcinogenesis pathways, which corroborates its potential *in vitro* anti-tumor effects. The present systematic review highlights the biological mechanisms of metformin used alone or together with traditional therapies for cancer. Though very limited, currently available preclinical evidence shows that metformin exerts a potential effect on head and neck carcinoma.

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Introduction

The association between cancer and metabolism has been extensively studied (1). While there are countless secondary characteristics, cancer cells are primarily characterized by aerobic glycolysis as opposed to oxidative respiration in normal cells, an event called 'Warburg effect' (2-5). Several drugs, including metformin, sulfonylureas and thiazolidinediones, have been shown to affect morbidity and cancer prognosis (5,6). The study of the therapeutic use of metformin in head and neck squamous cell carcinoma (HNSCC) treatment represents a new paradigm of clinical medicine (7). In addition, various studies have evaluated the use of metformin in combination with certain anti-cancer therapies (8-13).

Metformin (1,1-dimethylbiguanide hydrochloride) is a widely used drug for the treatment of type 2 diabetes mellitus (14,15), and it is currently considered one of the most widely prescribed drugs in the world (16). Only few adverse effects have been associated with its administration (17-20). Metformin use in diabetic patients has been associated with decreased cancer incidence and mortality (21-23). This effect seems to result from a reduction in circulating insulin levels (24), but there are also data indicating direct anti-tumor effects of metformin. The reduction in the growth of tumor cells in response to metformin is mediated, in part, by the inhibition of mammalian target of rapamycin complex 1 (mTORC1) (25), which is activated by adenosine monophosphate-activated protein kinase (AMPK) (25-27). The mTOR pathway plays a key role in the control of cell growth and metabolism, which are important in cancer progression (28). Carcinogenesis in HNSCC is driven by diverse signaling pathways, including epidermal growth factor receptor (EGFR), p53, p16, insulin growth factor (IGF) receptor, cyclin D1, human papillomavirus (HPV)/E6/E7, phosphoinositide 3-kinase (PI3K)/AKT/mTOR, nuclear factor kappa B and hypoxia-inducible factor 1 alpha (29). The associations between signaling pathways and metabolism have been extensively studied; however, little is known about the role of metabolism in HNSCC carcinogenesis, treatment failure and recurrence risk (30).

Mouth and pharynx cancers together are the sixth most common type of cancer in the world, with high incidence

particularly in the South and Southeast of Asia, parts of Western and Eastern Europe and parts of South America (31). The main risk factors for HNSCC are alcohol intake and tobacco smoking (32,33). Advancements in the management of HNSCC have included improved clinical care for these patients. The ultimate goal is to have therapies individually tailored to the specific genetic components of each patient (34). Therefore, the purpose of the present systematic review is to summarize the available literature about the *in vitro* anti-tumor effects of metformin on HNSCC.

Materials and methods

Protocol and registration. The present systematic review adheres as closely as possible to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses checklist (35). The protocol could not be registered as it is a systematic review of *in vitro* studies.

Eligibility criteria

Inclusion criteria. Only *in vitro* studies comparing the effects of metformin on the treatment of HNSCC cell lines were selected. The cell lines were established from different body parts affected by HNSCC, including the lips, oral cavity, pharynx, larynx, nasal cavity and paranasal sinuses (36). Studies on nasopharyngeal tumors were excluded due to differences in cancer etiology, epidemiology and therapeutic options. The population, intervention, comparison, outcome and study design format was adjusted to elucidate clinical questions based on the following inclusion criteria: i) Population, cells from HNSCC; ii) intervention, metformin; iii) comparison, cells that received a control treatment but not metformin treatment; iv) outcome, cell viability, apoptosis, cell cycle arrest and regulation of protein expression levels; and v) study design, studies with the presence or absence of a comparable baseline (*in vitro* studies), and randomized and non-randomized controlled trials (*in vivo* animal studies).

Exclusion criteria. i) Studies that did not treat cancer cells with metformin; ii) studies that did not establish an association between metformin and HNSCC; iii) previous reviews of the literature, letters, case reports, personal opinions, conference abstracts and book chapters; and iv) clinical studies were excluded from the present meta-analysis.

Information sources and search strategies. Studies to be considered for inclusion were identified by searching through multiple databases, including Cochrane Library (http://www.cochranelibrary.com), Embase (https://www.embase.com), LILACS (http://lilacs.bvsalud.org), MEDLINE(http://ovidsp.txovid.ez54.periodicos.capes.gov.br/sp) and PubMed (https://www.ncbi.nlm.nih.gov/pubmed/). The search strategy for PubMed included the following terms: 'Oral cancer' OR 'oral carcinoma' OR 'head and neck cancer' OR 'head and neck carcinoma' OR 'head cancer' OR 'head carcinoma' OR 'neck cancer' OR 'neck carcinoma' OR 'squamous cell carcinoma' AND metformin. Cited literature of the included studies was also checked. The search was performed on June 2, 2015. The references were managed manually and duplicate hits were discarded.

Study selection. Articles were selected in two phases. In phase 1, two authors (D.F.R. and S.T.E.) independently reviewed the titles and abstracts of all the references. These authors selected articles that appeared to meet the inclusion criteria based on their abstracts. In phase 2, two authors (D.F.R. and S.T.E.) read all full-text articles and excluded those that were not in agreement with the inclusion criteria. The same two authors independently reviewed all full-text articles. Any disagreement between the authors in the first and second phases was resolved by means of discussion. In cases where a consensus could not be reached, a third author (E.N.S.G.) made a final decision.

Data collection process and data items. One author (D.F.R.) collected the required information from the selected articles, including author names, year of publication, country, study design, assays, cell line, treatment used, results, main conclusions and clinical applications (Table I). A second author (S.T.E.) cross-checked the information. Any disagreement was resolved by means of discussion, and a third author (E.N.S.G.) was involved, when required, in making a final decision.

Risk of bias in individual studies. The authors methodically appraised all the selected studies according to the Grading of Recommendations Assessment, Development and Evaluation (GRADE) method (37) to judge the quality of evidence. Two authors (D.F.R. and S.T.E.) categorized the included articles as 'high', 'moderate', 'low' or 'very low' quality, according to their analysis of each study. When the above authors did not reach a consensus regarding the quality of a particular study, a third author (E.N.S.G.) made a final decision.

Summary measures. Cell viability, apoptosis, cell cycle arrest and changes in protein expression levels in HNSCC were the main evaluated outcomes of metformin treatment.

Synthesis of results. A meta-analysis was planned if the data from the included studies was considered relatively homogeneous.

Risk of bias across studies. Analysis of the risk of bias across studies was only applied if a meta-analysis was possible.

Results

Study selection. In phase 1 of study selection, 388 citations were identified across the aforementioned electronic databases. Following the removal of duplicate articles, 275 citations remained. Comprehensive evaluation of the abstracts was completed, and 248 articles were excluded. No additional studies from the reference lists were identified. The remaining 27 articles were retrieved to conduct a full text review. This process led to the exclusion of 16 studies (data not shown), resulting in the selection of 11 articles (7-13,30-41). A flow chart detailing the process of identification, inclusion and exclusion of studies is shown in Fig. 1.

Study characteristics. All included studies were published between 2011 and 2015, demonstrating that the use of metformin in HNSCC is a new concept. Nine of the

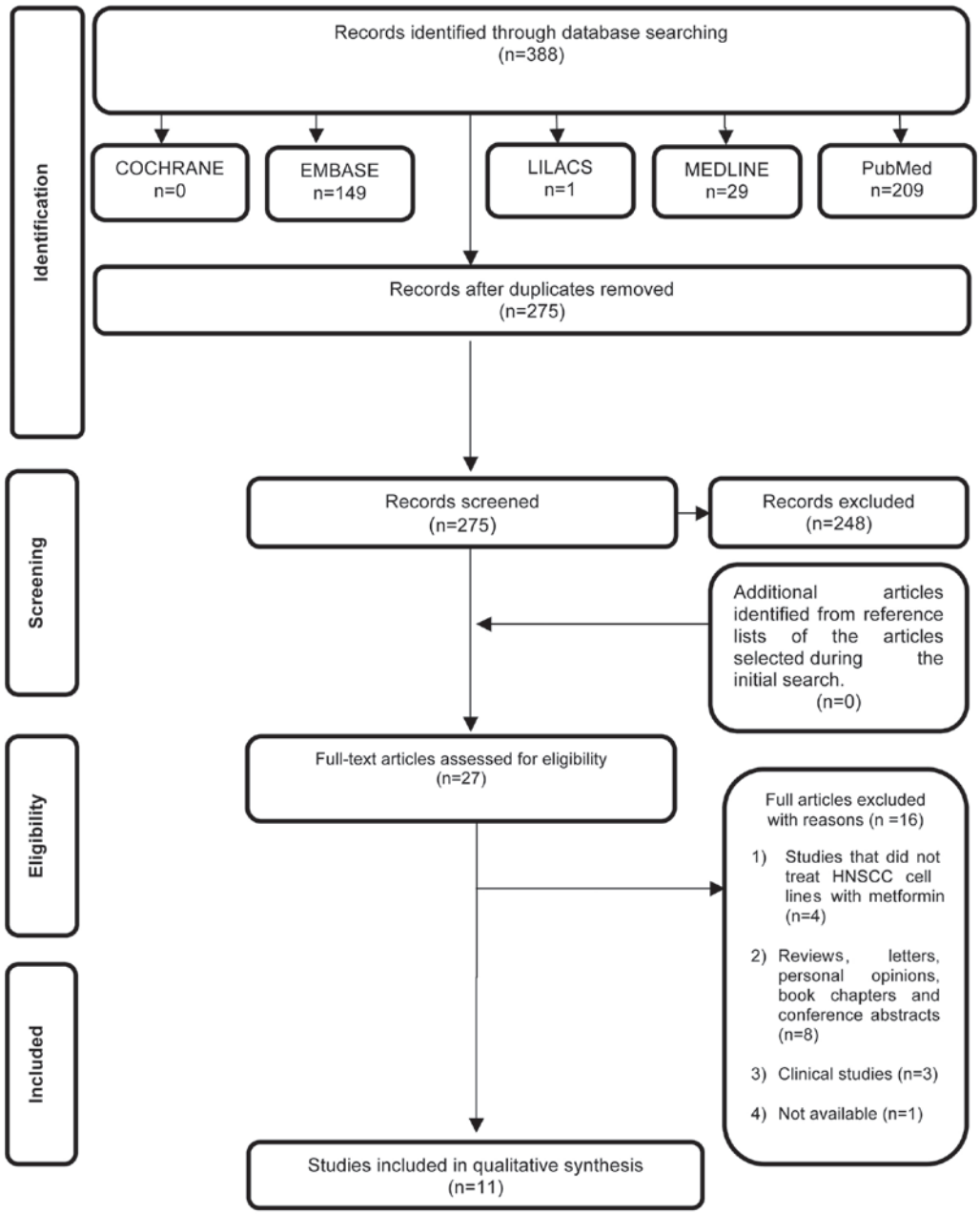

Figure 1. Flow diagram of literature search and selection criteria adapted from Preferred Reporting Items for Systematic Reviews and Meta-Analyses (34).

articles were published in English (7,10-13,39,40) and one in Chinese (41). The studies were conducted in four different countries: Canada (9), China (38,41), Taiwan (8) and USA (7,10-13,39,40). All selected studies were performed *in vitro* (7-13,38-41), and five of them involved *in vivo* animal experiments (7,8,13,38,41). A summary of the included studies is presented in Table I. The studies were compared according to the baselines for Randomized Controlled Trials (42), similarly to the work of Xiao *et al* published in 2013 (43), using the GRADE method (37). A summary of cell viability tests in HNSCC cell lines treated with metformin is shown in Table II.

Risk of bias within studies. The GRADE method was used to assess the quality of the included studies (37). Two studies were assigned a moderate quality (11,41). All others were considered high quality of evidence (7-12,13,38-40). One of these studies (11) did not describe the statistical methods used, and was considered inconsistent. Wang *et al* (41) used only one cell line (KB cells) to test the effect of metformin in HNSCC. Furthermore, the authors did not present a statistical analysis. Therefore, the results of the study were considered inconclusive (Table III).

The evidences describing a possibility of clinical application of metformin were classified as: i) 1, showing a potential effect following HNSCC treatment; ii) 2, inconclusive; or iii) 3, non-supportive of using metformin to treat HNSCC (Table I).

Cell viability. The cytotoxicity of metformin in HNSCC cells lines was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (7-10,38-41). Several studies monitored colony development (11,13).

Table I. Summary of the descriptive characteristics of the included articles (n=11).

Author, year (ref)	Country	Study design	Methods			Results	Main conclusion	Clinical application
			Assays	Cell line	Treatment			
Lin *et al*, 2014 (8)	Taiwan	*In vitro* and *in vivo*	MTT assay, flow cytometry and calculation of synergism between metformin and dasatinib and SEXTM	Ca9-22, HSC3, SAS and FaDu	Metformin+ dasatinib; DMSO (control)	Metformin and dasatinib for 48 h caused cellular growth inhibition; metformin enhanced dasatinib-induced apoptosis	Metformin enhanced dasatinib-induced apoptosis in sensitive HNSCC cells, while activation of AMPK by metformin potentiated dasatinib-induced endoplasmic reticulum stress, EGFR degradation and anti-tumor effect *in vivo*	1
Luo *et al*, 2012 (38)	China	*In vitro* and *in vivo*	Cell proliferation and clonogenic assay, cell cycle and apoptosis analysis, WB, IHC, TUNEL and *in vivo* anti-tumor activity	CAL27, WSU-HN6 and SCC25	Metformin; PBS (control)	Proportion of cells in the G0/G1 phase: 69.70 vs. 50.86% in CAL27, 77.96 vs. 56.54% in WSU-HN6 and 64.03 vs. 43.51% in SCC25 cells; colony formation was reduced >90% compared with the untreated controls	Metformin inhibited the growth of OSCC cells by blocking cell cycle progression at the G0/G1 phase and inducing apoptosis; metformin was associated with the activation of the AMPK pathway and suppression of mTOR and S6K activation, and markedly decreased the expression of cyclin D1 and increased the number of apoptotic cells in a xenograft model	1
Ma *et al*, 2012 (9)	Canada	*In vitro*	WB and MTT assay	SCC9 and SCC25	Metformin; metformin+ gefitinib	The combination of metformin and gefitinib induced co-operative cytotoxicity that was limited to the LKB1-expressing cell lines SCC9 and SCC25. In the SCC9 cell line, similar combinations of metformin or lovastatin with gefitinib displayed synergy when in combination	Metformin enhanced gefitinib cytotoxicity only in LKB1-expressing SCC lines	1

Table I. Continued.

Author, year (ref)	Country	Study design	Methods			Results	Main conclusion	Clinical application
			Assays	Cell line	Treatment			
Madera *et al*, 2015 (40)	USA	*In vitro* and *in vivo*	RNAi, OCT3 knockdown, IHC and WB	CAL27, CAL33 and UMSCC47	Metformin	Metformin inhibited mTOR signaling and tumor growth in HNSCC cells expressing mutated PIK3CA and HPV oncogenes, which required OCT3 expression	Metformin reduced the proliferation *in vitro* of HNSCC cells harboring mutations in PIK3CA or derived from HPV^+ HNSCC lesions, which are frequent events in oral malignancies. Metformin was highly effective in reducing HNSCC tumor growth *in vivo*	1
Patel *et al*, 2013 (10)	USA	*In vitro*	Cell viability, RNAi and WB	HN4, HN13 and Hep2	Metformin; metformin+ corticosterone	OCT3 is highly expressed in oral dysplastic lesions and well to moderately differentiated HNSCC tumors. Therefore, metformin was unable to induce AMPK activation or inhibit the mTORC1 pathway	The impact of OCT3 on metformin action was defined a novel chemopreventive oncologic agent of head and neck cancer	1
Sandulache *et al*, 2012 (11)	USA	*In vitro*	Metabolic studies, clonogenic assay and ROS measurement	HN30 and HN31	Metformin+ 2-DG; metformin+ 2-DG+XRT	In combination with 2-DG, metformin resulted in potentiation of XRT toxicity. Metformin triggered phosphorylation of AMPK but did not induce an increase in the levels of pAMPK	Inhibition of respiration using metformin increased glycolytic dependence in wt TP53-expressing cells and potentiated the effects of glycolyic inhibition on radiation toxicity	1
Sandulache *et al*, 2011 (12)	USA	*In vitro*	Soft agar growth	FaDu, HN30, OSC19, HN31, SQCCY1, PCI13, UMSCC17A, UMSCC22B, UMSCC17B, MDA1586, SCC61 and UMSCC25	Metformin+ 2-DG	Addition of metformin (a glucose sensitizer) resulted in substantial potentiation of 2-DG effects in multiple cell lines, independently of p53 mutation status	Metformin greatly potentiated the effects of glycolytic inhibition irrespective of p53 status	1

Table I. Continued.

Author, year (ref)	Country	Study design	Methods			Results	Main conclusion	Clinical application
			Assays	Cell line	Treatment			
Sikka *et al*, 2012 (39)	USA	*In vitro*	Cell viability assay, cell cycle analysis and WB	FaDu and D562	Metformin; DMEM (control)	Metformin inhibited cell growth and cell cycle progression, decreased the protein levels of CDKs, CDKIs, cyclins and oncogenic proteins SKP2 and β-TrCP, decreased 4E-BP1 phosphorylation, and increased EF2 and AMPK phosphorylation	Metformin suppressed cell growth through targeting global translational regulators in two different human HNSCC cell lines	1
Skinner *et al*, 2012 (13)	USA	*In vitro* and *in vivo*	Clonogenic assay, immuno fluorescence, ROS measurement, WB, cell cycle analysis and p21 transcription orthotopic mouse model	HN30, UMSCC and UMSCC17A	Metformin	Metformin selectively radiosensitized cells with disruptive TP53 mutations, partially due to altered senescence	Metformin could serve as a radiosensitizer for HNSCC with disruptive TP53 mutations	1

Table I. Continued.

Author, year (ref)	Country	Study design	Methods			Results	Main conclusion	Clinical application
			Assays	Cell line	Treatment			
Vitale-Cross *et al*, 2012 (7)	USA	*In vitro* and *in vivo*	Cell proliferation and viability assay, ATP assay, WB, experimental animal model, plasma levels of IGF1 and insulin, IHC, immuno fluorescence, T-cell proliferation assay and flow cytometry	CAL27, HN12, HN13 and Hep2	Metformin; rapamycin (control)	Metformin treatment inhibited HNSCC cell proliferation, downregulated mTORC1 pathway activity through an AMPK-independent mechanism and prevented HNSCC development by significantly reducing the size and number of carcinogen-induced oral tumoral lesions and by preventing their spontaneous conversion to squamous cell carcinomas	Metformin could become an attractive chemopreventive agent to hamper the progression of premalignant lesions highly dependent on mTORC1 activity	1
Wang *et al*, 2014 (41)	China	*In vitro*	MTT assay, flow cytometry and WB	KB	Metformin	Metformin inhibited HNSCC cell proliferation and KB clone colony formation. Metaformin promoted the apoptosis and increased the expression of GRP78 and caspase-3 protein in the oral cancer KB cell line	Metformin significantly inhibited the proliferation of human oral cancer KB cells, and induced apoptosis, the mechanism of activation of the mitochondrial apoptotic pathway and excessive endoplasmic reticulum stress. This finding suggests that metformin may be used as a novel adjuvant and used to treat cancer	1

Clinical application was classified by the present authors from the analysis of results of the use of metformin as: i) 1, potential effect in HNSCC treatment; ii) 2, inconclusive; and iii) 3, evidence not supportive as a drug to HNSCC treatment. ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide; IHC, immunohistochemistry; HNSCC, head and neck squamous cell carcinoma; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OCT3, organic cation transporter 3; PBS, phosphate-buffered saline; SEXTM, subcutaneous ectopic xenograft tumor model; TUNEL, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling; WB, western blotting; AMPK, adenosine monophosphate-activated protein kinase; EGFR, epidermal growth factor receptor; OSCC, oral squamous cell carcinoma; S6K, S6 kinase; LKB1, liver kinase B1; RNAi, RNA interference; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; HPV, human papilloma virus; ROS, reactive oxygen species; 2-DG, 2-deoxyglucose; XRT, X-ray radiotherapy; pAMPK, phosphorylated AMPK; wt, wild type; TP53, tumor protein p53; DMEM, Dulbecco's modified Eagle's medium; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; SKP2, S-phase kinase-associated protein 2; β-TrCP, β-transducin repeat-containing protein; 4E-BP1, 4E-binding protein 1; EF2, elongation factor 2; IGF, insulin growth factor; GRP78, 78 kDa glucose-regulated protein.

Table II. Interventions used to test head and neck carcinoma cell lines viability in cell culture.

Author, year (ref)	Population	Intervention						
	Cell line[a]	Treatment	Time (h)	Dose	C	O	S	V
Lin *et al*, 2014 (8)	Ca9-22, HSC3, SAS and FaDu	Metformin; dasatinib; metformin+dasatinib; DMSO (control)	48	0.5-10 mM metformin; 0.05-1 μM dasatinib	√	√	√	1
Luo *et al*, 2012 (38)	CAL27, SCC25 and WSU-HN6	Metformin; PBS (control)	24,48 and 72	0-20 mM metformin	√	√	√	1
Ma *et al*, 2012 (9)	SCC9 and SCC25	Metformin, lovastatin; gefitinib; lovastatin+ gefitinib; ethanol (control)	24,48 and 72	0-20 mM metformin; 0-100 μM lovastatin; 0-100 μM gefitinib	√	√	√	1
Madera *et al*, 2015 (40)	CAL27, CAL33 and UMSCC47	Metformin	4	0-03 mM metformin	√	√	√	2
Patel *et al*, 2013 (10)	HN4, HN13 and Hep2	Metformin	72	3 mM metformin	√	√	√	2
Sandulache *et al*, 2012 (11)	HN30 and HN31	Metformin+2-DG; metformin+2-DG+XRT	16	5 mM metformin; 5 mM 2-DG; 2 Gy XRT	√	√	√	2
Sandulache *et al*, 2011 (12)	HN30 and HN31	Metformin; 2-DG; metformin+2-DG	72	1 mM metformin; 0.5-8 mM 2-DG	√	√	√	2
Sikka *et al*, 2012 (39)	FaDu and D562	Metformin	24 and 72	5-20 mM metformin	√	√	√	1
Skinner *et al*, 2012 (13)	HN30, UMSCC and UMSCC17A	Metformin+XRT; PBS (control)	24	5-10 μmol/l metformin; 4 Gy XRT	√	√	√	1
Vitale-Cross *et al*, 2012 (7)	HN12	Metformin; rapamycin (control)	24 and 96	2-100 mM metformin	√	√	√	2
Wang *et al*, 2014 (41)	KB	Metformin	24,48 and 72	1.25-20 mmol/l metformin	√	√	√	1

[a]Head and neck squamous cell carcinoma immortalized or primary cell lines. C, control; O, outcomes; S, study (randomized controlled trial or comparable baselines); √, yes; V, percentage of cell viability (1, 0-49% of viable cells; 2, 50-100% of viable cells); PBS, phosphate-buffered saline; XRT, X-ray radiotherapy; 2-DG, 2-deoxyglucose; DMSO, dimethyl sulfoxide.

Table III. Judgment of the quality of evidence for intervention.

	Grading of Recommendations Assessment, Development and Evaluation factors								
Author, year (ref)	Study design	Study limitation	Inconsistency	Indirectness	Imprecision	Publication bias	Moderate/ large effect size	Dose effect	Overall quality
Lin *et al*, 2014 (8)	With comparable baseline (*in vitro*)	√	√	√	√	√	Present	Present	++++
Luo *et al*, 2012 (38)	With comparable baseline (*in vitro*) RCT (animal)	√	√	√	√	√	Present	Present	++++
Ma *et al*, 2012 (9)	With comparable baseline (*in vitro*) RCT (animal)	√	√	√	√	√	Present	Present	++++
Madera *et al*, 2015 (40)	With comparable baseline (*in vitro*) RCT (animal)	√	√	√	√	√	Present	Present	++++
Patel *et al*, 2013 (10)	With comparable baseline (*in vitro*)	√	√	√	√	√	Present	Present	++++
Sandulache *et al*, 2012 (11)	With comparable baseline (*in vitro*)	√	X (No statistical analysis for all results)	√	√	√	Present	Present	+++
Sandulache *et al*, 2011 (12)	With comparable baseline (*in vitro*)	√	√	√	√	√	Present	Present	++++
Sikka *et al*, 2012 (40)	With comparable baseline (*in vitro*)	√	√	√	√	√	Present	Present	++++
Skinner *et al*, 2012 (13)	With comparable baseline (*in vitro*)	√	√	√	√	√	Present	Present	++++
Vitale-Cross *et al*, 2012 (7)	With comparable baseline (*in vitro*)	√	√	√	√	√	Present	Present	++++
Wang *et al*, 2014 (41)	With comparable baseline (*in vitro*)	√	√	√	X (Only one cell line used; no statistical analysis)	√	Present	Present	+++

Overall quality of evidence (+, very low; ++, low; +++, moderate; ++++, high); √, no serious limitations; X, serious limitations (or not enough information present to know effect size and dose effect). Unclear, unable to rate item based on available information; RCT, randomized controlled trial.

Six studies (8,9,38-41) showed that metformin alone is cytotoxic to HNSCC cells, reducing cell viability by >50% in a dose-dependent manner (Table II). Other studies involved co-administration of metformin with another drug, and demonstrated an adjuvant effect of metformin (8,10-12). Lin *et al* (8) observed that metformin enhanced dasatinib efficacy through activation of AMPK. Sandulache *et al* (11) demonstrated the anti-tumorigenic activity of 2-deoxyglucose (2-DG) when combined with metformin, resulting in the potentiation of radiation toxicity, despite minimal single agent effects. Sandulache *et al* (12) showed in multiple cell lines that this process was independent of tumor protein p53 status. Ma *et al* (9) compared the ability of metformin or lovastatin to enhance the cytotoxicity of gefitinib in various liver kinase B1 (LKB1)-deficient cell lines, and demonstrated that metformin increased the cytotoxic effects of gefitinib in the LKB1-expressing cell lines SCC9 and SCC25, which were derived from tongue squamous cell carcinoma. Specifically, the authors observed >90% cell death in SCC9 cells treated with both metformin and gefitinib. Madera *et al* (40) observed that metformin significantly reduced cell proliferation in CAL27, CAL33 and UMSCC47 cell lines. The HPV^+ UMSCC47 cell line exhibited the strongest sensitivity to metformin, with a decrease in cell proliferation at lower than CAL33 metformin concentrations, in 3H-thymidine incorporation and colony forming assays. It was reported that metformin significantly decreased colony size in colony forming assay (40). Two studies (11,13) demonstrated beneficial effects of metformin when combined with radiotherapy.

Cell cycle regulation and apoptosis. Three studies (8,38,39) demonstrated accumulation of cells in the G0/G1 phase upon treatment with metformin, as evidenced by flow cytometry assays of HNSCC cell lines. Lin *et al* (8) demonstrated metformin-dependent enhancement in the apoptotic activity of dasatinib in Ca9-22 and HSC3 cells, suggesting that metformin could potentiate the dasatinib-induced anti-cancer effect. Luo *et al* (38) showed that metformin increased the proportion of cells in the G0/G1 phase in three HNSCC-derived cell lines, compared with control cells (69.70 vs. 50.86% in CAL27, 77.96 vs. 56.54% in WSU-HN6 and 64.03 vs. 43.51% in SCC25 cells). In addition, metformin induced a significant increase in the proportion of apoptotic tumor cells 48 h after treatment in CAL27, WSU-HN6 and SCC25 cells (25.4, 24.4 and 43.7%, respectively, compared with 11.4, 8.4 and 15.5% of apoptotic cells at 24 h after treatment, respectively). Sikka *et al* (39) reported a dose- and time-dependent increase in G1-phase cell population in FaDu and D562 cell lines ($P<0.001$) following treatment with metformin.

Regulation of protein expression level. Lin *et al* (8) demonstrated metformin-dependent enhanced effects of dasatinib on the phosphorylation of AMPK and elongation factor 2, in addition to down-regulation of EGFR, in sensitive HSC3 tumor cells. According to Sikka *et al* (39), metformin affects the expression of cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs) in human HNSCC cells 24 and 48 h after treatment, causing a strong and dose-dependent decrease in the expression levels of cyclins D1 and E in FaDu and D562 cell lines. Luo *et al* (38) confirmed the role of metformin in the expression of related cell cycle regulatory proteins (including AMPK, mTOR, S6 kinase, cyclin D1, retinoblastoma protein, CDKs 4 and 6, p21 and p27) in arrested and proliferating oral squamous cell carcinoma cells. The expression of the anti-apoptotic protein B-cell lymphoma (Bcl)-extra large and the pro-apoptotic protein Bcl2-associated X protein were also regulated by metformin. Patel *et al* (10) reported the abrogation of increased S6 and acetyl coenzyme A carboxylase phosphorylation levels, as well as the inhibition of mTORC1 in metformin-treated HN13 cells transfected with organic cation transporter 3 (OCT3) small interfering RNA (siRNA), compared with control siRNA-transfected cells. These findings indicate that, in HNSCC cells, the uptake transporter OCT3 plays a key role in mediating the intracellular effects of metformin on AMPK activation and subsequent inhibition of mTORC1 activity. Sandulache *et al* (11) demonstrated that, in combination with 2-DG, metformin triggered the phosphorylation of AMPK, whereas alone, metformin did not increase the levels of phosphorylated AMPK. Vitale-Cross *et al* (7) observed that, in the absence of AMPK activation, metformin treatment led to a marked decrease in mTORC1 activity and tumor cell proliferation. Madera *et al* (40) revealed a reduction in phosphorylated S6 levels upon treatment with metformin in xenograft tumor models, whereas the non-phosphorylated fraction of 4E-binding protein 1 (4E-BP1) increased, indicating a cumulative decrease of 4E-BP1. In alignment with their known activities, treatment with metformin resulted in increased phosphorylated AMPK levels, while both treatments diminished phosphorylated S6. Thus, metformin downregulates the activity of the mTOR signaling pathway in HNSCC cell lines *in vitro*. Similarly, the reduction of OCT3 diminishes the metformin effect on cell proliferation *in vitro* and its anti-tumoral effect *in vivo*. According to Wang *et al* (41), metformin caused an increase in activated caspase-3 expression levels, as well as an initial up-regulation followed by a down-regulation of 78 kDa glucose-regulated protein expression in KB cells.

Risk of bias across studies. The studies selected for the present analysis were considered heterogeneous, and they did not have compatible data that would allow a meta-analysis. In addition to the non-comparability of the results of each study, a meta-analysis could not be conducted due to a lack of clinical studies on the subject of interest.

Discussion

Summary of evidence. The present systematic review evaluated the *in vitro* and *in vivo* (animals) anti-tumor effects of metformin in HNSCC. The majority of HNSCC cases arise in the oral cavity, and continue to be a major public health concern (31). Despite the use of multiple treatments, the prognosis for this aggressive solid tumor remains poor (44). Surgery is the most well established initial treatment for the majority of oral cancers; however, radiotherapy is employed in conjunction with surgery (45). Considering organ preservation and survival, a multidisciplinary approach is strongly encouraged, and concurrent chemo-radiotherapy has been recommended (46). Complete response rates subsequent to induction chemotherapy combined with radiotherapy are

usually higher than following radiotherapy alone. In addition to high nodal stage, molecular mechanisms should be identified and integrated in order to elucidate markers of distant metastasis risk (47). The review from Busch *et al* (47) highlights the recent developments, including randomized trials, comparing radiotherapy and induction chemotherapy, followed by definitive radiotherapy. The review summarizes the developments in induction chemotherapy, provides critical remarks of recent discoveries and discusses how clinical trials such as those assessing induction chemotherapy should be conducted in the future (46). There is a requirement for novel perspectives and therapeutic approaches to provide a better understanding and more successful treatment of HNSCC (47).

Metformin is a biguanide that has been used for its insulin-sensitizing and glucose-lowering effects in type 2 diabetes mellitus, as well as in gestational diabetes mellitus, polycystic ovary syndrome and metabolic syndrome, and also for diabetes prevention (48). This drug has shown beneficial effects in suppressing the intestinal absorption of glucose (17), reducing cardiovascular risk (19), reducing the risk of lactic acidosis compared with other biguanides (20), in addition to anti-neoplastic activity *in vitro* and *in vivo* (49,50). Numerous studies have demonstrated the anti-tumor effect of metformin in cancer progression (6,15,51-53). A recent publication confirmed the association between decreased risk of HNSCC and metformin use in clinical studies (54). Therefore, metformin, an old drug, is now gaining increasing attention as an anti-cancer agent (55).

The mechanisms underlying the possible anti-cancer effect of metformin have not yet been fully elucidated. Data from clinical and pre-clinical studies suggest that amelioration of insulin resistance/hyperinsulinemia and glucose lowering by metformin may play a role, since both the insulin-IGF1 system (56-58) and hyperglycemia (59-61) have been associated with cancer risk. However, there has been mounting evidence of metformin's direct effects on cancer cells, and promising models have been proposed, which suggested the inhibition of cell proliferation and progression of cancer, cell cycle arrest and stimulation of apoptosis (62). In the present systematic review, the *in vitro* effect of metformin on HNSCC was studied, and 11 *in vitro* studies addressing this topic were identified (7-13,38-41). To evaluate the effects of metformin on HNSCC cell lines, the authors used MTT assay (7-10,38-41) or colony formation tests (11,13), and demonstrated that metformin alone or in combination with other treatments could effectively reduce cancer cell viability by >50%, in a time- and dose-dependent manner (Table II). The effect of metformin on HNSCC cells in combination with radiation was also evaluated (11,13), and was demonstrated to sensitize the tumor to the effect of radiotherapy.

Combination of metformin with other drugs inhibits cancer cell proliferation (8,11). Metformin has been reported to improve cancer responses to radiation therapy, probably via down-regulation of the hyperactive PI3K/AKT/mTOR signaling pathway (63). Previous studies have reported that mTOR plays a key role in controlling cell growth, proliferation and metabolism, and mediates the PI3K/AKT signaling pathway, which is frequently dysregulated in human cancers (64,65).

Multiple genetic changes leading to cancer progression cause dysregulation of the G1 to S transition (66). The G1 phase of the cell cycle is controlled by a dynamic interaction between cyclins D1 and E, CDKs 2, 4 and 6 and CDKIs, including members of the CDK interacting protein/kinase inhibitory protein (Kip) and inhibitors of CDK4 (67). During the transition from G1 phase, the levels of Kip1/p27 decrease to allow the cyclin/CDK complex to initiate the transcription of genes necessary for G1-S progression (68). Lin *et al* (8), Luo *et al* (38) and Sikka *et al* (39) demonstrated accumulation of cells in the G0/G1 phase when HNSCC cell lines were treated with metformin. Activation of AMPK and subsequent inhibition of mTORC1 signaling, reduction of cyclin D1 levels and dephosphorylation of AKT (at Ser473) have been shown to participate in metformin-induced apoptotic process (69).

In summary, the present study is the first systematic review of the *in vitro* anti-tumor effects of metformin in HNSCC cell lines, and the first review to summarize the evidence of a positive association between the decrease of HNSCC cell viability and metformin. The present authors have previously published a systematic review about the clinical effects of metformin on HNSCC patients (55), which suggested that metformin appears to improve the overall survival of HNSCC patients. Considering the context, multiple mechanisms of metformin action should therefore be clarified, and the current review summarizes the potential effects of metformin on such important pathways of HNSCC carcinogenesis as cell proliferation, cell cycle progression and protein expression.

Limitations. While the present review has methodological limitations, which should be considered, its main strength lies in the description of several studies that tested metformin in combination with other drugs. Despite the fact that the included studies were aimed at understanding the *in vitro* effects of metformin on the expression of proteins involved in the process of carcinogenesis of HNSCC, only three studies described the effect of metformin in G0/G1 cell cycle arrest and apoptosis. Therefore, further investigations are warranted to validate these findings. For the quality assessment of *in vitro* studies, no standard assessment was used for basic studies; however, a method to assess the quality of all articles was defined.

In conclusion, the present systematic review reveals that preclinical evidence supports the potential use of metformin as an adjuvant agent in chemotherapy and/or radiotherapy approaches routinely used in the management of HNSCC. The aforementioned studies demonstrated that metformin is important in the inhibition of cell proliferation, G0/G1 cell cycle arrest, apoptosis and regulation of various proteins involved in cancer pathways, thus corroborating its potential *in vitro* and *in vivo* (animals) anti-tumor effects. Based on these data and its favorable safety profile, the present authors suggest the future use of metformin in both molecular and clinical trial studies.

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