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Research Article
Metformin Induces Cytotoxicity in Oral Squamous Cell Carcinoma Cells by Targeting CCN1/Akt-Axis

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Abstract

Background and Objective: Oral cancer contributes to one of the highest cancer-related deaths worldwide. Diabetes has been linked to an increased risk of developing several malignancies, including oral cancer. The current study focuses on how an antidiabetic drug, Metformin, shows anticancer properties on oral cancer cells. The matrix-associated protein Cyr61, also known as CCN1 has been known to be a positive regulator of oral cancer. Hence this study investigated whether metformin can target CCN (Cyr61) signalling in oral cancer SCC25 cells. **Materials and Methods:** The effect of metformin on the viability of SCC25 cells was evaluated by MTT assay. Expression of CCN1 in SCC25 cells was depleted by transfecting the cells with CCN1 si-RNA, using Lipofectamine® 3000. In addition, the expression profile of CCN1, Akt, p-Akt and β-Actin was monitored by western blot. **Results:** Metformin treatment significantly reduced cell viability in oral squamous cell carcinoma cells, SCC-25, in a dose-dependent as well as, a time-dependent manner. Metformin, on the other hand, had a minimal inhibitory effect on the non-cancerous HEK-293 cell line. Metformin treatment reduced the Cyr61 expression, both dose-dependently and time-dependently, in OSCC cells.Cyr61-siRNA was used as a positive control to show Cyr61 down-regulation inhibits the PI3K/AKT pathway in SCC25 cells. Metformin had a similar effect in SCC25 cells. **Conclusion:** This suggests that Cyr61 acts as an upstream regulator of the PI3K/AKT pathway. Furthermore, metformin was found to inhibit the PI3K/AKT/Cyr61 cascade by lowering Cyr61 and pAKT expression. Therefore, all these findings indicate that metformin decreased cell viability in the SCC25 cell line by targeting PI3K/AKT/Cyr61.

Key words: Anticancer, metformin oral cancer, CCN1 (Cyr61), Akt

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oral Cancer (OC) is one of the leading causes of cancer-related deaths on earth[1]. Oral Squamous Cell Carcinoma (OSCC) is the type of OC that is commonly found, accounting for 91% of all OC cases[2]. Overall, the OSCC accounts for 1-2% of all human cancers[1]. Surgical resection in combination with or without radiation or chemotherapy is currently used for OC treatment. But both conventional chemotherapeutic therapy and surgical resection are found to be ineffective and recovery is mainly determined by the stage of the recurring tumor[3]. Therefore, developing a novel treatment strategy for treating OSCC is essential.

According to multiple epidemiological studies, diabetes has been linked to an increased risk of several malignancies, including oral cancer[4]. Diabetes is also connected to a poor prognosis and survival rate in individuals suffering from oral cancer[5]. Hence oral cancer treatment may benefit from the use of antidiabetic medications that also have anti-cancer properties. An oral biguanide medicine, metformin, predominantly used to treat type 2 diabetes has exhibited anticancer potential against a variety of cancers[6-8]. Metformin is currently being explored in clinical trials to treat several cancers[9,10]. Metformin also has been reported to be associated with better clinical outcomes for patients with OSCC[11]. However, the mechanism by which metformin may inhibit the growth of OSCC cells is not well explored.

The matrix-associated protein Cyr61, also known as CCN 1, is a cysteine-rich angiogenic inducer. It is a member of the CCN family of growth factors (Cyr61/CTGF/Nov). Cry61 is involved in a wide range of cellular functions, including proliferation, differentiation, adhesion, migration, invasion and angiogenesis[12]. Cyr61 is known to be over-expressed in various cancers, including oral cancer[13,14]. Furthermore, Cyr61 has been associated with poor clinical outcomes in different cancer types[15]. Metformin has been known to attenuate the invasive properties of pancreatic cancer cells by inhibiting CCN1 signaling[16]. However, no such evidence has been observed in the case of oral cancer. Furthermore, Cyr61 has been reported to modulate the PI3K/Akt cascade in cancer[17]. This PI3/AKT cascade is the most significant regulator of various cancer hallmarks, which include proliferation, autophagy, metastasis, angiogenesis, cell-survival, invasiveness and epithelial-to-mesenchymal transition (EMT)[18]. Several reports suggest that Akt may be a downstream modulator of CCN1[19,20] but there is no such report in oral cancer. This study investigated that how metformin restricts the oncogenic potential of oral cancer cells by targeting the Cyr61/Akt axis.

MATERIALS AND METHODS

Study area: The study has been conducted within one year, from 01.05.2020-30.04.2021.

Cell culture: Human oral squamous carcinoma cells (SCC25) and human embryonic kidney 293(HEK293) cells were procured from the American Type Culture Collection (ATCC, USA). SCC25 and HEK293 cells were grown at 37°C in a humidified chamber in RPMI 1640 complete medium supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics such as penicillin (100 U mL^{-1}) and streptomycin (100 g mL^{-1}).

Cell viability assay: The SCC25 and HEK293 cells were seeded in a 96-well plate and exposed to different concentrations of metformin for various time points (24, 48 and 72 hrs). The dose and time-dependent antiproliferative effect of metformin against the SCC25 and the HEK293 was determined using the crystal violet assay[21].

Gene silencing using small interfering RNA (siRNA): Cyr61-specific siRNA and scrambled control siRNA were purchased from Santa Cruz Biotechnology. SCC25 cells were transfected using the Lipofectamine® 3000 reagent (Thermo) in OptiMEM (Thermo), following the manufacturer's protocol[22].

Western blot analysis: The SCC25 cell extract was obtained using a RIPA buffer after ZA treatment. Western blot was used as a tool to analyze both treated and untreated cells, to determine the expression of several proteins such as Cyr61, AKT and pAKT. Dilutions of primary antibodies were prepared according to the manufacturer's instructions. For loading controls, β-actin was used. Bradford's method was used to calculate the protein concentration[23].

Statistical analysis: The Mean±Standard Deviation (SD) was used to express all data. Statistically significant differences between groups were established using the student's t-test for two-sample comparisons and the one-way ANOVA followed by Tukey's post hoc test for more than two-sample comparisons. Statistical analysis of various experimental data was done by using the GraphPad Prism 7 software (GraphPad Software, USA). A value of P determined that is <0.05, was considered statistically significant.

RESULTS

Metformin showed both time-dependent and dose-dependent cytotoxicity against OSCC cells in a selective manner: The impact of metformin in Fig. 1a on the growth of the non-cancerous cell line HEK-293 and the human oral squamous carcinoma cell SCC25 was examined by growing the cells and treating them with varying doses of metformin for different periods (24, 48 and 72 hrs). Metformin effectively suppressed SCC25 proliferation in a time and dose-dependent manner in Fig. 1b. OD_{600} values plotted on the Y-axis are a representation of the viable cells that were stained by crystal violet. After a 72 hrs incubation period, the IC_{50} value was determined to be 10 mM from the graph. Furthermore, metformin showed minimal effect on the viability of the non-cancerous HEK-293 cell line in Fig. 1c. At 10 mM concentration of metformin, only a 15% decrease in viable HEK-293 cells was observed after 72 hrs of treatment.

CCN1 (Cyr61) acted as a direct target of metformin in cancer cells: To study whether Cyr61 is a direct target of metformin measured Cyr61 expression levels in metformin-treated SCC25 cells over time and at different doses. In metformin-treated SCC25 cells detected a dose-dependent in Fig. 2a and b and a time-dependent in Fig. 2c and d reduction in Cyr61 expression. When compared to the untreated control, 5 and 10 mM concentrations of metformin decreased Cyr61 expression in SCC25 cells by around 4 and 5-folds, respectively, after 48 hrs in Fig. 2a and b. After 48 hrs, 10mM metformin reduced Cyr61 expression by almost 4fold as compared to the control in Fig. 2c and d.

Depletion of CCN1 (Cyr61) attenuates the viability of SCC25 cells: The effects of Cyr61-specific siRNA on SCC25 cell viability were studied to determine whether Cyr61 may be a suitable therapeutic target for OSCC. To validate the efficiency of Cyr61-specific siRNA did a Western blotting

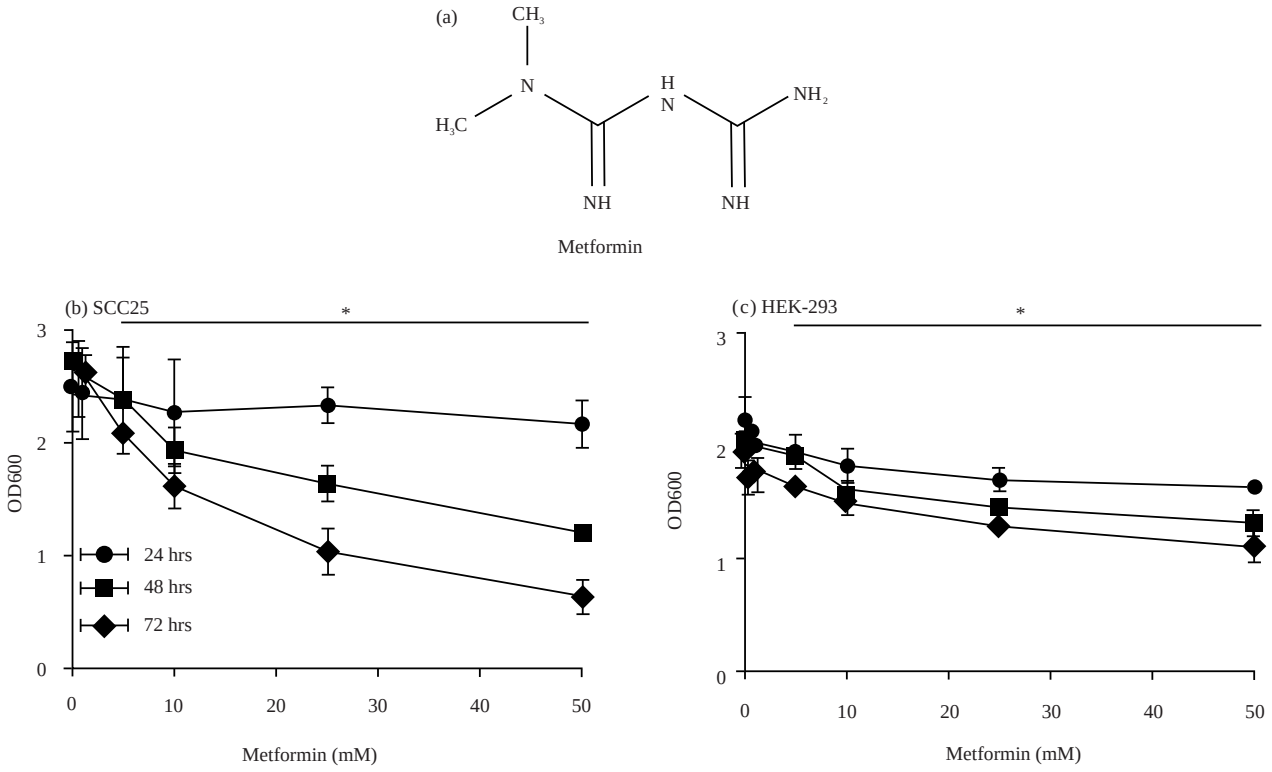

Fig. 1(a-c): Effect of metformin on cell viability, (a) Chemical structure of metformin, (b) Effect of metformin on cell viability of oral squamous cells carcinoma cells SCC25 and (c) Effect of metformin on cell viability of non-cancerous cells HEK-293

Reduced viability of oral squamous cells carcinoma cell line (SCC25) was measured by crystal violet assay in the presence of various concentrations of metformin and at different time points, the reduced viability of non-cancerous cell line (HEK-293) was measured by MTT assay in the presence of various concentrations of Z-ajoene. All the data reported are a Mean±SEM of three experiments performed independently. *p<0.05 compared to data from untreated cells

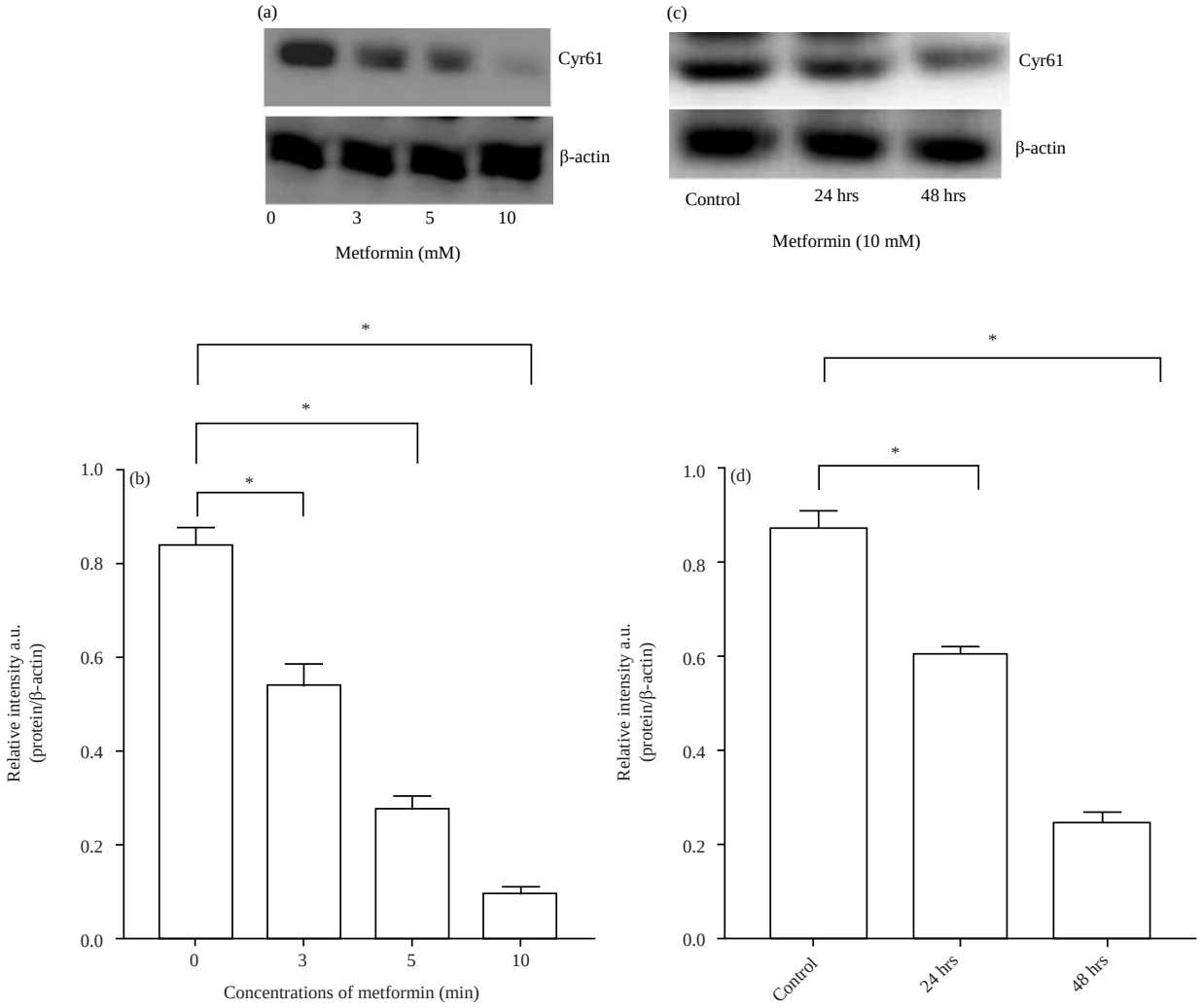

Fig. 2(a-d): Effect of metformin on expression levels of Cyr61 in SCC25 cells, (a) Western blot analysis of Cyr61 expression in various concentrations of metformin-treated SCC25 cells, (b) Graphical representation of protein bands after densitometric quantification using ImageJ (NIH software), (c) Western blot study of Cyr61 expression in SCC25 cells treated with 10 mM metformin for 24 and 48 hrs and (d) Graphical representation of protein bands after densitometric quantification using ImageJ (NIH software) for 24 and 48 hrs

All the data reported are a Mean±SEM of three experiments performed independently. *p<0.05 compared to data from untreated cells, equal amounts of total cellular protein of various concentrations of metformin-treated SCC25 were analyzed by immunoblotting using respective antibodies, equal amounts of total cellular protein of 10 mM of metformin-treated SCC25 were analyzed by immunoblotting using respective antibodies

investigation. Cyr61 protein expression was considerably reduced when Cyr61-specific siRNA was transfected into SCC25 cells in Fig. 3a and b. Following the role of Cyr61 in the survival of the SCC25 cell line, a considerable decrease in cell viability was observed in the cells that were transfected with Cyr61-siRNA, in comparison to cells that were transfected with scrambled siRNA in Fig. 3c. This data implies that Cyr61 is a necessary protein for SCC25 cell survival and that its reduced expression causes cell death.

CCN1 (Cyr61) is an upstream regulator of PI3K/Akt signalling: This study performed western blot analysis to explore the effect of reduced expression of Cyr61 on PI3K/AKT signalling. In Cyr61-siRNA-transfected SCC25 cells, the expression of pAKT was significantly down-regulated compared to scrambled-siRNA transfected cells in Fig. 4a and b. In contrast, AKT protein expression remains unchanged in both Cyr61-siRNA and scrambled-siRNA transfected SCC25 cells in Fig. 4a and b. This

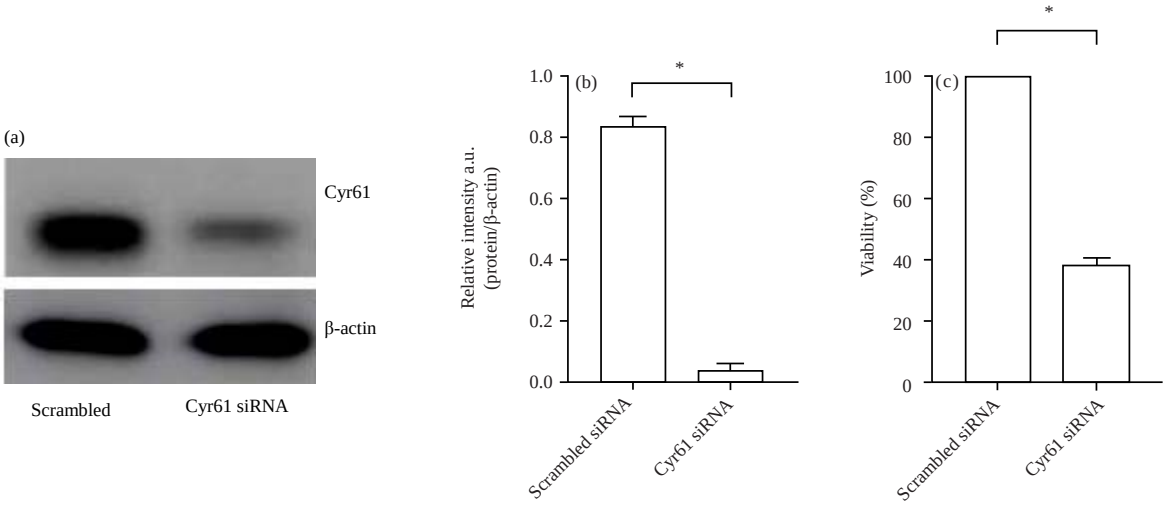

Fig. 3(a-c): CCN1 (Cyr61) promotes viability of SCC25 cells, (a) Western blot analysis of expression of Cyr61 in scramble siRNA and Cyr61 siRNA treated SCC25 cells, (b) Graphical representation of Cyr61 protein bands after densitometric quantification using ImageJ (NIH software) and (c) Reduced viability of the SCC25 cell line was measured by crystal violet assay in the presence of scrambled siRNA and Cyr61 siRNA

All the data reported are a Mean±SEM of three experiments performed independently. *p<0.05 compared to data from untreated cells. An equal amount of total cellular protein of scramble siRNA and Cyr61 siRNA treated SCC25 cells were analyzed by immunoblotting using respective antibodies

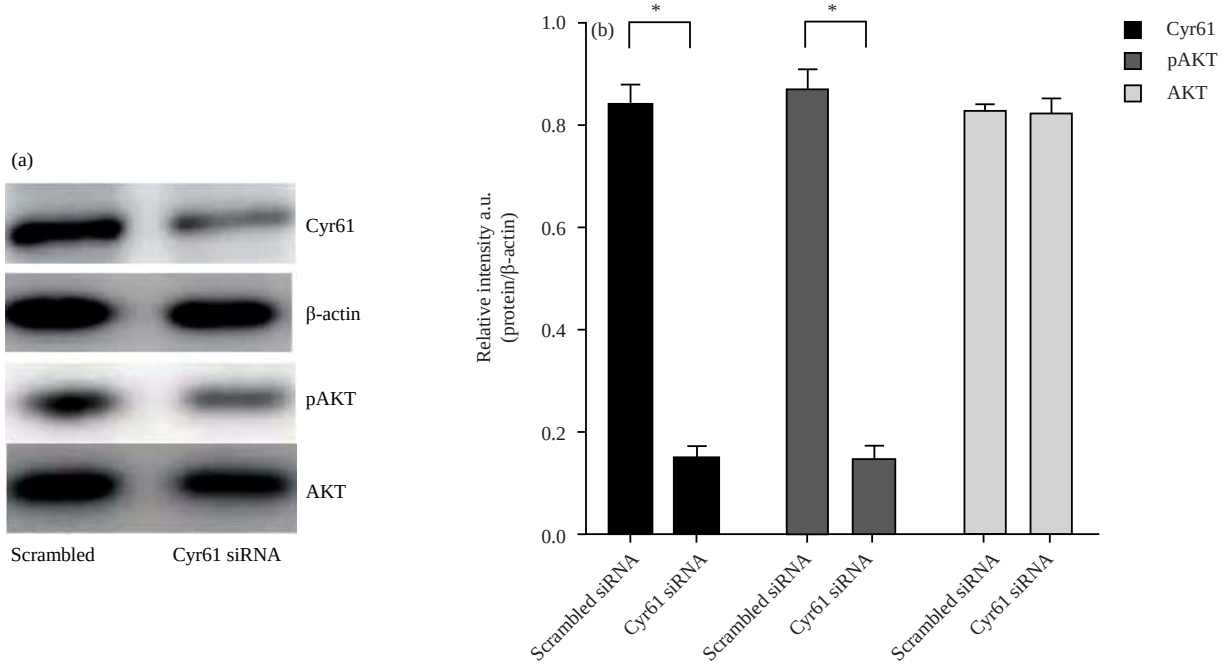

Fig. 4(a-b): CCN1 (Cyr61) modulates pAKT/AKT pathway, (a) Western blot analysis of expression of Cyr61, pAKT, AKT in scramble siRNA and Cyr61 siRNA treated SCC25 cells and (b) Graphical representation of Cyr61, pAKT, AKT protein bands after densitometric quantification using ImageJ (NIH software)

All the data reported are a Mean±SEM of three experiments performed independently. *p<0.05 compared to data from untreated cells. An equal amount of total cellular protein of scramble siRNA and Cyr61 siRNA treated SCC25 cells were analyzed by immunoblotting using respective antibodies

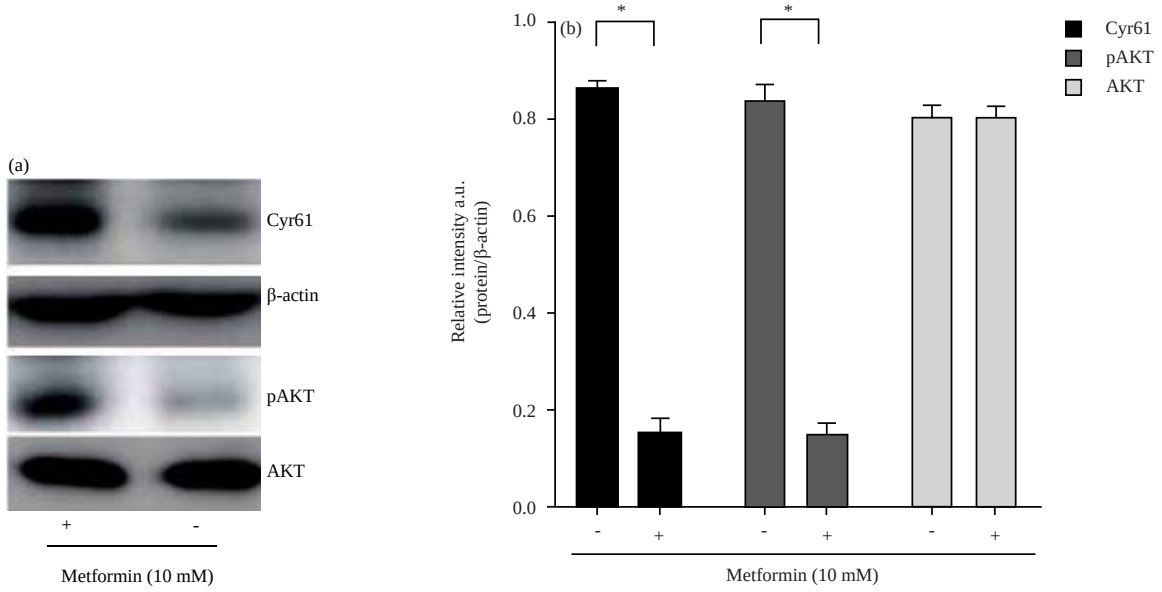

Fig. 5(a-b): Metformin inhibits Cyr61/pAKT/AKT axis, (a) Western blot analysis of expression of Cyr61, pAKT, AKT in SCC25 cells in presence and absence of 10 mM metformin and (b) Graphical representation of Cyr61, pAKT, AKT protein bands after densitometric quantification using ImageJ (NIH software)

All the data reported are a Mean±SEM of three experiments performed independently. *p<0.05 compared to data from untreated cells. An equal amount of total cellular protein of 10 mM metformin-treated and untreated cells were analysed by immunoblotting using respective antibodies

finding suggests that CCN1 (Cry61) functions as an upstream regulator of the PI3/AKT pathway in oral cancer cells.

Metformin inhibits the CCN1/Akt axis in SCC25 cells: To study the effect of metformin on PI3K/AKT signalling, measured the levels of pAKT and AKT expression in metformin-treated SCC25 cells. Notably, metformin treatment resulted in a considerable decrease in pAKT levels in SCC25 cells in Fig. 5a and b. However, the overall level of AKT expression remained unchanged in Fig. 5a and b. Simultaneously Cyr61 expression was considerably reduced in cells treated with 10 mM metformin for 72 hrs. Hence, all of these findings indicate that metformin substantially decreased the PI3/AKT/Cyr61 axis in oral cancer cell lines, demonstrating its antiproliferative action.

DISCUSSION

The PI3K/AKT cascade is associated with the growth, progression and metastasis of various human cancers, including oral cancer[18]. Furthermore, the PI3K/AKT pathway is important for the generation of chemoresistance features and the poor prognosis of oral cancer[24,25]. Cyr61, a member of the CCN family of growth factors, on the other hand, is highly overexpressed in oral cancer cells and is associated with the poor outcome of the disease[13-15]. CCN1 is involved in several processes in oral cancer cells, including proliferation, adhesion, invasion, migration and angiogenesis[12]. Furthermore, Cyr61 has been shown to regulate the PI3K/AKT pathway in a variety of cancer cells[17]. As a result of all of this information, it is possible to postulate that the CCN1/Akt axis is a potential therapeutic target in oral cancer. Despite recent technological advances, only minor improvements in oral cancer survival have been achieved and these are primarily due to early detection rather than treatment measures[26]. Hence, the development of new drug candidates for the prevention of ovarian cancer has become an essential demand.

Das *et al.*[16] have reported that metformin, an antidiabetic medicine, has anti-cancer activity against pancreatic cancer cells via inhibiting CCN1 signalling. However, metformin has also been reported to be effective against oral cancer cells[11]. However, the particular mechanism of action of this drug in oral cancer cells remains unknown. Hence, the current investigation, intensively investigated whether Cyr61 as an upstream modulator of the PI3K/AKT pathway, serves as a direct target for metformin.

According to current findings, metformin treatment resulted in a considerable reduction of cell viability in oral cancer cells SCC25 in both a dose and time-dependent manner (Fig. 1b). However, in the non-cancerous HEK-283 cell line, there was the relatively little inhibitory impact of metformin (Fig.1c). These findings suggest that metformin has a selective inhibitory effect on OSCC cells. Metformin administration resulted in a dose- and time-dependent reduction in Cyr61 expression in OSCC cells (Fig. 2a-d). This study investigated the function of Cyr61 in the cell viability of the SCC25 cell line. This study observed that knocking down Cyr61 with SiRNA resulted in SCC25 cells losing their survivability (Fig. 3c). Furthermore, Cyr61-siRNA suppresses the PI3K/AKT pathway in SCC25 cells (Fig. 4a and b). This indicates Cyr61 acts as an upstream regulator of the PI3K/AKT pathway. Moreover, metformin was also found to inhibit the PI3K/AKT/Cyr61 cascade by decreasing Cyr61 and pAKT expression (Fig. 5a and b). Therefore, all of these findings indicate that metformin decreased cell viability in the SCC25 cell line by targeting PI3K/AKT/CCN1.

Metformin targeting the PI3K/AKT/CCN1 axis in oral cancer cells has been shown by us for the first time. Earlier reports suggest an anti-malignant role of metformin on OSCC cells[27-29]. Metformin also has an anti-proliferative role on oral cancer cells[30]. These findings are similar to the effect of metformin found on SCC25 cells. It is previously known that metformin downregulates CCN1[16], which is also reflected in current findings.

Because it is widely acknowledged that diabetic individuals have a very high chance of acquiring cancer, metformin can be used to treat both disease conditions. Although this study only used one cell model, future research employing many more cell lines and *in vivo* assessment of metformin would explore metformin as an anti-cancer agent in the treatment of oral cancer.

CONCLUSION

The study mainly focuses on the anticancer property of Metformin, an antidiabetic drug, on oral squamous cell carcinoma cells (SCC-25). Metformin resulted in a significant reduction in the viability of SCC-25 cells by downregulating CCN1 (Cyr61) protein expression, which in turn downregulates the PI3K/AKT pathway. Diabetes, in many instances, is related to the development of different forms of cancer. Thus metformin, a well-known anti-diabetic, which can also show anticancer properties, can prove to be a better choice of treatment in oral cancer.

SIGNIFICANCE STATEMENT

This study discovered the role of Metformin, an antidiabetic drug, showing anti-cancerous properties that can be beneficial for the treatment of oral cancer. Also, the target signalling was identified, which added novelty to this study. This will help the researchers to uncover better treatment options for OSCC.

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