Metformin Down Regulate Mcl-1 and Induce Apoptosis of Human Oral Cancer Cells Through MiR-26a

Chong Zhang1**, Xueqin Bai1**, Hongwei Liu2, Qingxia Xu1

1Department of Pathology, Jining First People’s Hospital, Jining, 272100, China
2Department of Stomatolgy, Jining First People’s Hospital, Jining, 272100, China

*Corresponding author: Qingxia Xu, Email: mianmian2009@126.com
**Chong Zhang and Xueqin Bai contribute equal to this article as co-first author

Abstract
In this paper, the effect of metformin on apoptosis of human oral cancer KB cells was studied. After treated with metformin for 24 hours, the growth and morphological changes of KB cells were observed under inverted microscope. MTT method was used to detect the cell survival rate, plate cloning was used to observe the effect of metformin on the colony forming ability of oral cancer cells in vitro; after KB cells were treated in groups, mitochondrial membrane potential test kit was used to detect the change of mitochondrial membrane potential in cells; after miR-26a was transfected, colony cloning test and PI single staining test were used to detect the overexpression of miR-26a in KB cells, metformin was used to detect the overexpression of miR-26a in KB cells. After that, the expression of Mcl-1 was detected by Western blot. Compared with the control group, metformin can significantly up regulate the expression of miR-26a in cells. The results showed that compared with metformin alone, the proliferation of KB cells was significantly inhibited, while the inhibition of miR-26a showed the opposite results. Further experiments showed that the Mcl-1 level of KB cells transfected with miR-26a decreased significantly, and the apoptosis rate increased significantly. Metformin can significantly inhibit the proliferation of KB cells and induce apoptosis; up regulation of Mcl-1 can reduce the sensitivity of KB cells to metformin; the effect of metformin on the proliferation and apoptosis of oral cancer may be related to the up regulation of miR-26a by metformin.

Key words: Oral cancer; Metformin; Apoptosis; Mcl-1; miR-26a

1. Introduction

Oral cancer is one of the most common malignant tumors in the head, neck and maxillofacial region, accounting for 3-5% of the total malignant tumors. Squamous cell carcinoma is the most common, accounting for more than 90%. Tumor related gene changes (including the activation of proto oncogene and the inactivation of tumor suppressor gene) may lead to the uncontrolled regulation of cell proliferation and / or apoptosis, which may be the main mechanism of oral cancer[1]. Secondly, the occurrence of oral cancer is closely related to the
changes of host microenvironment, including genetic susceptibility, immune status of the body and DNA damage and repair ability[2-3]. The occurrence of oral cancer will not only affect the appearance of patients, but also cause some psychological problems. It will also cause chewing, swallowing, breathing and voice disorders, and seriously reduce the quality of life of patients[4]. At present, the treatment of oral cancer is still mainly surgery, and multi-disciplinary comprehensive treatment is advocated. However, most of the patients with oral cancer have been diagnosed as advanced or distant metastasis, and it is difficult to achieve the ideal prognosis only by surgical resection, and may produce new complications[5-6]. Therefore, it is a new direction of cancer treatment research to seek new sensitive drugs, explore the related targets of drug resistance mechanism in the treatment process of malignant tumors, and improve the clinical treatment effect[7].

In this study, oral cancer cells are the main research objects, and metformin has been proved to have inhibitory effect on a variety of tumor cells. On the one hand, different concentrations of metformin act on KB cells of oral cancer, through the detection of cell proliferation and apoptosis related indicators, to observe the effect of metformin on KB cells of human cavity cancer, providing strong evidence for the anti-tumor effect of metformin. On the other hand, the relationship between metformin and microRNA and the enhancement of the sensitivity of microRNA to apoptosis induction in tumor cells were further studied, which provided new ideas and targets for tumor treatment.

2. Materials and methods

2.1 Human oral cancer cell lines and drugs
(1) Human oral epithelial cancer cell line Kb: purchased from Shanghai cell bank. Culture and cryopreservation in the laboratory of Biochemistry and pharmacology, Bengbu Medical College.
(2) Metformin: purchased from sigma company in the United States

2.2 Annexin V / PI double staining flow cytometry to detect apoptosis
2.2.1 Inoculated cells
KB cells were inoculated into 6-well cell culture plate with a density of 10 × 10⁴ / ml, 1 ml per well. After the cells adhered to the wall, 1 ml fresh medium was added to each well, and then they were cultured in the incubator for routine culture, and the solution was changed once every two days. When the cells grow to 70% - 80% confluence, they are divided into groups according to the experimental requirements.

2.2.2 Kb cell treatment
According to the experimental design, the MEM culture solution with different concentrations of metformin was replaced. After 24 hours, the cell culture solution was sucked out to a suitable centrifuge tube. The centrifuge tube was placed on ice, the cells were washed with precooled PBS for 1-2 times, and the washing solution was transferred to the corresponding grouped centrifuge tube. Add an appropriate amount of 0.25% trypsin to the 6-well plate and put it into the incubator to digest the cells until they are suspended. Pay attention that the digestion time should not be too long, otherwise it will affect the binding of phosphatidylserine and annexin V on the cell membrane. Pour the previously collected cell solution into the corresponding hole, mix gently to stop digestion. Centrifuge 2000r · min⁻¹, centrifuge for 10min, and discard the supernatant. Cells were resuspended with precooled PBS and counted. Take an appropriate amount of cells (50000 cells in this experiment), 2000r · min⁻¹, centrifugation for 10min, use the sampling gun to suck up the supernatant of cells as much as possible, immediately add 195 lannexin V-FITC binding solution to the centrifuge tube, and gently flick the bottom of the tube with fingers to make the cells completely mixed. Add 5 lannexin V-FITC to the cell solution, do not add it to the wall of the tube, mix it gently with a straw, and then incubate it at room temperature in dark for 15 minutes. The above cell solution was centrifuged at 2000r · min⁻¹ for 5min, and the supernatant was aspirated as much as possible with the sampling gun. Add 190 lannexin V-FITC and tap the tube wall to make the cell mass mix well. Finally, 10 L propidium iodide staining solution was added into the above system. After gently blowing and mixing with a straw, it was placed in an ice bath and kept away from light with tin foil paper. The flow cytometry was used for detection within 1h.

2.2.3 Flow cytometry
The fluorescence of FITC was detected by 488nm, 515nm passband filter and 560nm filter respectively.

2.2.4 Result judgment
In the early stage of apoptosis, PS on cell membrane can combine with annexin V labeled by FITC to produce fluorescence signal. In the later stage of apoptosis, when the cell membrane is damaged, the DNA in the cell is stained by pi to produce red fluorescence. Therefore, on the scatter diagram of bivariate flow cytometry, the first quadrant represents the damaged cells (an PI +) during cell collection, the second quadrant represents
the late apoptotic cells (an + PI +), the third quadrant represents the normal living cells (an pi -), and the fourth quadrant represents the early apoptotic cells (an + pi -).

2.3 miRNA transient transfection

2.3.1 Inoculated cells

The KB cells with good growth condition were inoculated into 6-well cell culture plate with a cell density of 2 × 10^5 / ml, 2ml per well. It is suitable to carry out the experiment when the fusion degree of cells was 60% ~ 70% from the day of conventional culture to the day of transfection (no more than 24 hours).

2.3.2 Transfected gene

According to the instructions of lipofectaminetm2000, mix the transfection and the opti MEM medium gently at 1:50, then let it stand at room temperature for 5min, then dilute the lipofectaminetm2000 liposome with 50 times of the opti MEM medium, mix it gently, incubate it at room temperature for 5min, finally let it stand at room temperature for 20min to form the transfection complex, and then add the mixture to the serum free and antibiotic free medium Cells were cultured in cell medium for 6 hours and then replaced with MEM for 24 hours. Cells were collected for subsequent experiments.

2.4 Statistical analysis

Each experiment was repeated at least 3 times, and all data in the experiment were expressed by mean ± standard deviation. SPSS software was used for data analysis and graphpad prism 5.0 was used for statistical mapping. T-test was used to compare the data of the two groups, and P < 0.05 was defined as statistically significant difference.

3. Results

3.1 Effect of metformin on the morphology and growth of KB cells

The cell morphology was observed by inverted phase contrast microscope, as shown in Figure 1: KB cells in logarithmic growth stage were placed in the incubator after cell passage. About 4hkb cells began to adhere to the wall. After the cells adhered to the wall, they grew in a polygonal shape, with full shape, strong refraction, close connection between cells and unclear outline[8]. When the cells in the culture flask grew to 80% fusion degree and were interfered by different concentrations of metformin for 24 hours, compared with the control group, some of the cells treated by metformin began to become round and small, the boundary was fuzzy, the cell membrane was transparent, the refraction was strong, the adherent cells were reduced, and a small number of cells began to float[9-10]. With the increase of metformin concentration, the above phenomenon is more obvious. A large number of floating cells were found in 20mmol · L⁻¹ group 24 hours later.

![Figure 1](attachment:image.png)

**Figure 1.** Comparison of cell morphology between the two groups under inverted phase contrast microscope
A control group (×200); B 5mmoL· L⁻¹ group; C 10mmoL· L⁻¹ group; D 20mmoL· L⁻¹ group (×200)

According to the results of MTT, KB cells were stimulated with one tenth of the inhibition concentration, and the inhibitory effect of metformin on the proliferation of oral cancer cells was detected by colony-clone staining. It can be seen from Figure 2 that metformin can inhibit the formation of cell colony clone, and the inhibition rate tends to increase with the increase of drug concentration[11].
3.2 Effect of metformin on KB cell apoptosis

The results of MTT and colony cloning showed that metformin could inhibit the proliferation of KB cells. We further used annexin V-FITC / PI double staining kit to detect the apoptosis of oral cancer cells after metformin intervention. KB cells were treated with high concentration of metformin (5, 10, 20mmol · L⁻¹) for 24 hours. Annexin V-FITC / PI staining was carried out after the cells were collected. Figure 3 shows that compared with the group without drugs, the cell apoptosis rate increased with a certain concentration of metformin (P < 0.05). With the increase of metformin concentration, the apoptotic rate of cells increased gradually[12-13].

3.3 Mechanism of apoptosis of KB cells induced by metformin

3.3.1 Metformin reduces mitochondrial membrane potential of KB cells

In the experiment, we have confirmed that metformin can inhibit the proliferation of KB cells and induce apoptosis. In order to explore its preliminary anti-tumor mechanism, we first used JC-1 fluorescence probe to detect the effect of metformin on the membrane potential of KB cells. When the cells were not stimulated by apoptosis, the mitochondrial membrane potential was normal. At this time, JC-1 monomer gathered in the mitochondrial matrix, forming a polymer that can produce red fluorescence. On the contrary, when the mitochondrial membrane potential is reduced, JC-1 is a monomer that produces green fluorescence. Therefore, the change of JC-1 from red fluorescence to green fluorescence can be used as an indicator to detect early apoptosis. From the laser confocal scanning picture in Figure 4, it can be seen that the cells in the control group present bright red fluorescence, without obvious green fluorescence, indicating that the cells in the control group ΔΨ m are normal, while metformin is used In the treatment group, the red fluorescence intensity decreased significantly, while the green fluorescence intensity increased significantly, which indicated that metformin
caused a significant decrease of $\Delta \psi_m$ in oral cancer cells. It can be speculated that early apoptosis of cells occurred.

**Figure 4.** Effect of metformin on mitochondrial membrane potential of KB cells

3.3.2 Effect of metformin on the expression of mitochondrial apoptosis pathway related proteins

Secondly, Western blot was used to detect the effect of metformin on the expression of apoptosis related proteins in KB cells. In the experiment, we used 10 mmol $\cdot$ L$^{-1}$ metformin to intervene KB cells of oral cancer at different times (0, 6, 16, 24h). Western blot was used to detect the expression of Mcl-1, Bax and BIM. From the results of the experiment (Fig. 5), we can see that the expression of Mcl-1, the classical anti apoptotic factor in the apoptotic mitochondrial pathway, decreased with the increase of time, while the expression of Bax and BIM, the corresponding Pro apoptotic factors, decreased. The expression of KB cells was up-regulated, which led to the apoptosis of KB cells.

**Figure 5.** Expression of apoptosis related proteins in KB cells of oral cancer

3.4 The effect of over expression of miR-26a on the clonogenesis of oral cancer cells

Firstly, mir-26amic and inhibitor were transiently transfected into the cells, then digested and adjusted to a density of $1 \times 10^4$ in 6-well plates, cultured in a 37 °C, saturated humidity, 5% CO2 incubator for 24 hours, and stimulated with 0.5 mmol $\cdot$ L$^{-1}$ metformin at a concentration of 0.5 mmol $\cdot$ L$^{-1}$. The inhibitory effect of miR-26a on the proliferation of oral cancer cells was detected by clonal staining. It can be seen from Figure 6 that compared with the control cells, the number of cell clones formed by miR-26a transfection decreased significantly, while the number of cell clones after miR-26a transfection did not decrease significantly compared with the control cells.
Figure 6. The effect of over expression of miR-26a on the colony forming ability of KB cells in oral cancer

4. Discussion

Apoptosis, also known as programmed cell death (PCD), refers to an active and highly ordered death process in which a series of enzymes are involved and controlled by genes when cells are stimulated by adverse factors in vitro and in vivo. Abnormal apoptosis can lead to a variety of diseases, including malignant tumors, neurodegenerative diseases, autoimmune diseases and virus infection[14]. The formation of tumor in vivo is that under the stimulation of various oncogenic factors, the cell proliferation is out of control at the gene level, and abnormal differentiation occurs. At the same time, the occurrence and development of tumor is closely related to the imbalance of tumor cell apoptosis[15-16]. In the clinical treatment of tumor, one of the main mechanisms of common methods such as chemotherapy, radiotherapy, hormone therapy, hyperthermia and some biotherapy is to increase the ratio of cell death / proliferation by inducing apoptosis of tumor cells, so as to inhibit the development of tumor and play an anti-tumor role. Therefore, it is an effective tumor treatment strategy to intervene the apoptosis of tumor cells by taking the imbalance of apoptosis as the starting point[17].

The occurrence of apoptosis has a very complex regulatory mechanism. According to the different sources of apoptotic signals, apoptotic signal transduction pathways are mainly divided into death receptor pathway, mitochondrial pathway and endoplasmic reticulum pathway, which are related to each other and jointly regulate apoptosis. At the same time, with the development of research on the molecular mechanism of apoptosis, many important events in cells are closely related to mitochondria. To some extent, it can also be considered that mitochondria are the active center of apoptosis regulation. It is of great theoretical significance to study the mechanism of mitochondria in apoptosis. When cells are stimulated unfavourably, it will lead to the opening of mitochondrial permeability transition pore (MPTP) between the inner and outer membrane of mitochondria, the disappearance of transmembrane potential (δ ψ m), the production of superoxide ions, the outflow of Ca2 + in matrix and the release of some inter membrane proteins. In turn, the production and release of these substances can promote MPTP to enter One step opening, forming a cascade reaction, eventually leading to the activation of caspase (cysteine aspartate specific proteinase) family protein, resulting in lethal cell results. In this study, annexin V-FITC / PI results showed that metformin can induce apoptosis of KB cells, suggesting that metformin may be a new antitumor drug[18-19].

Mcl-1 (myeloidcell leukemia-1) is one of the important members of Bcl-2 family and plays an important role in the signal transduction pathway of apoptosis. It has been found that the abnormal expression and regulation of Mcl-1 is related to many human diseases, and Mcl-1 can be used as an effective prognosis / prediction index in the clinical treatment of diseases. Mcl-1 protein in cells can be located on different cell intima, so as to regulate the most important response in the process of mitochondrial apoptosis in a timely and effective manner[20]. Mcl-1 interacts with the BH3 domain of the pro apoptotic protein BIM or Bax through its surface hydrophobic structure to form heterodimer, which prevents Mcl-1 from forming pore on the mitochondrial membrane, maintains the stability of the mitochondrial membrane, reduces the release of cytochrome c to the cytoplasm, and inhibits apoptosis. Therefore, Mcl-1 plays an important role in cell survival and apoptosis. In our study of KB in oral cancer, we found that after metformin intervention, the expression of Mcl-1 protein decreased significantly, while the expression of Bim and Bax increased, thus promoting the apoptosis of tumor cells. In order to verify this result, we transfected cdnamcl-1 into KB cells, which increased the expression of Mcl-1 protein in KB cells of oral cancer[21]. After treatment with metformin, we found that the proliferation of KB cells was not significantly inhibited after overexpression of Mcl-1. Similarly, after overexpression of Mcl-1 protein, the expression of Bax and BIM was inhibited by metformin, and caspase-3
was inhibited. Their activity was also inhibited. These results suggest that metformin may play an important role in the regulation of KB cell proliferation and apoptosis by inhibiting the expression of Mcl-1[22].

MicroRNA, also known as small interfering RNA, is a kind of small molecular RNA encoded by endogenous non-coding genes with a length of about 21-25 nucleotides, which is involved in post-transcriptional horizontal regulation of genes. It combines with the 3’ untranslated region (3’UTR) of the target gene. Through the specific gene silencing mediated by miRNA, target mRNA degradation or inhibition of protein synthesis are caused, regulating the expression of post-transcriptional genes involved in the regulation of cell development, differentiation, metastasis and apoptosis. MiRNA is an attractive drug target, and there are specific miRNA deletions or down-regulation in many solid tumors. It was found that the abnormal expression of miRNAs is closely related to the occurrence, development and prognosis of oral cancer. It has been reported that miR-26a can directly induce apoptosis by acting on the target gene Mcl-1. Therefore, over expression of miR-26a can promote apoptosis of tumor cells, on the contrary, it can protect cell survival. In order to further verify the role of miR-26a in the apoptosis of KB cells induced by metformin, we first transiently transfected mir-26amic and inhibitor, respectively, through qRT-PCR. The results of colony cloning showed that the ability of cell proliferation decreased significantly after transient transfection of mir-26amic with metformin compared with that of cells stimulated by metformin alone, while the opposite result was obtained after silencing with mir-26aminhibitor. In order to further verify whether miR-26a can determine cell survival by regulating the expression of Mcl-1, Western blot showed that transient transfection of mir-26amic and inhibitor over expressed and interfered with miR-26a, respectively, after treatment with the same concentration of metformin, miR-26am was transfected in IMIC group, the expression of Mcl-1 protein was significantly down regulated, which was consistent with the previous results. At the same time, the flow cytometry results further confirmed our hypothesis. The above results showed that over expression of miR-26a could inhibit the proliferation of oral cancer cells in vitro, and play an important role in the mechanism of metformin anti-cancer, but the detailed mechanism is still unclear.

5. Conclusion

Metformin can significantly inhibit the proliferation of KB cells and induce apoptosis; up regulation of Mcl-1 can reduce the sensitivity of KB cells to metformin; up regulation of miR-26a after metformin stimulation of KB cells; the effect of metformin on the proliferation and apoptosis of oral cancer can be related to up regulation of miR-26a by metformin.

References


