ASSOCIATION OF MIRNA23A AND GLUCOSE TRANSPORTERS IN DIABETIC ORAL SQUAMOUS CELL CARCINOMA

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Abstract

Introduction: Tumor cells are characterized by abnormally accelerated energy metabolism. Therefore, inhibition of energy metabolism is considered a promising therapeutic target for cancer treatment. Glucose transporter (GLUT) is a uniporter protein which facilitates the transport of glucose in human cells. MicroRNAs (miRNAs) are small (20-22nt), tissue specific, non-coding RNA molecules which cause mRNA translational inhibition or degradation by binding to complementary target mRNAs. Global down-regulation of miRNA expression is an apparent feature of various human tumors, including Oral squamous cell carcinoma (OSCC). The aim of the study is to analyse the association of miRNA23a and Glucose transporters in diabetic OSCC

Materials and methods: Samples of Control (n=5) , Precancerous tissue (n=5), Carcinoma tissue (n=5) were collected. miRNA was isolated from control and experimental samples using TRIR (total RNA isolation reagent) kit. Diluted RNA sample was quantified spectrophotometrically by measuring the absorbance (A) at 260/280 nm. RT-PCR is an approach for converting and amplifying a single stranded RNA template to yield abundant double stranded DNA products.The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. 2X Reaction buffer, Forward primer, Reverse primer , cDNA- Template, Autoclaved milliQ water. Human GLUT 1 gene specific primers used were FW-5'-TGTGCAACCCATGAGCTAA-3'and RW-RW-5' CCTGGTCTCATCTGGATTC-3'.

Results: Overexpression of miRNA-23a was identified with increased fold change in OSCC. miRNA-23a promotes tumor cell differentiation and glucose metabolism in OSCC by acting as an upstream activator of GLUT1.

Conclusion: miRNA-23a was identified to be up-regulated in OSCC. miRNA-23a may promote cancer cell proliferation and glucose uptake in OSCC by acting as an upstream activator of GLUT1.

Keywords: GLUT, miRNA, Squamous cell carcinoma, Upregulation

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1. Introduction

Oral squamous cell carcinoma (OSCC) has emerged to be the tenth most common cancer in the world with 275,000 estimated incidence annually and increasing mortality rates (1). 90% of all malignant oral lesions are Oral squamous cell carcinomas (OSCC), which are more common in young and middle-aged people (2). Amongst the various types of malignant cancer occurring in the hard palate, the upper and lower alveolar ridges, retromolar trigone, the two-thirds of the tongue, submucosal region, buccal mucosa and floor of the mouth, oral squamous cell carcinoma has high incidence with poor prognosis in India (3). The occurrence of OSCC has been demonstrated by a multi-storey progression which is significantly influenced by poor diet, poor oral hygiene, smoking, chewing of tobacco, alcohol consumption and human papillomavirus infection (4). Oral carcinogenesis is the disruption of the normal regulatory pathways, which allows the functioning of basic cellular events that include cell division, differentiation, cell death and alteration of normally functioning tumor suppressor genes and oncogenes (5).

Tumor cells are characterized by abnormally accelerated energy metabolism. Therefore, inhibition of energy metabolism is considered a promising therapeutic target for cancer treatment (6). Glucose transporter (GLUT) is a uniporter protein which facilitates the transport of glucose in human cells. Studies suggest that GLUT1 expression is a prognostic and diagnostic marker in tumors (7). Previous studies have been reported that GLUT1 is abnormally upregulated in malignant tumors which helps in the cancer development and progression by regulating the cancer cell glucose metabolism (8,9). Evidence has indicated that miRNAs regulate malignant progression of OSCC. However, to our knowledge, the molecular mechanism of the etiology of oral cancer remains unclear, hampering the development of effective therapeutic strategies.

MicroRNAs (miRNAs) are an important element in cancer biology. MicroRNAs are involved in certain cases of cancer biology by affecting energy metabolism, especially by regulating the expression of GLUT1 (10). MicroRNAs (miRNAs) are small (20-22nt), tissue specific, non-coding RNA molecules which cause mRNA translational inhibition or degradation by binding to complementary target mRNAs (11). In microRNAs, miR-23a belongs to the cluster of miR-23~24~27. Previous research shows overexpression of miR-23a which inhibits migration, invasion of osteosarcoma cells and reduces their differentiation (12).

Previous investigation shows that miR-23a inhibited epithelial-mesenchymal transformation (EMT) in endometrioid adenocarcinoma which is in contrast that miR-23a acts as an oncogene in gastric cancer (13). The long-term survival of cancer patients remained poor due to a high risk of locoregional recurrence and new malignant conversions, despite the fact that treatment techniques such as surgical resection, radiotherapy, and chemotherapy had markedly enhanced over the the past few decades. Reliable and innovative prognostic markers are absolutely necessary in order to accurately identify high-risk patients and further determine clinical outcomes (14). However, the role of miR-23a in Oral squamous cell carcinoma requires further study on target drug therapeutics in cancer treatments. Our team has extensive knowledge and research experience that has translate into high quality publications (15–24))

This study reveals that miRNA-23a can promote cancer cell proliferation in oral squamous cell carcinoma (OSCC), by functioning as an upstream activator of GLUT1 and promoting glucose metabolism.

2. Materials and methods

Sample collection

The tissue samples for the study were collected from Oral squamous cell carcinoma patients with diabetes who reported to the Oral surgery department of Saveetha Dental College and Hospitals, Chennai. The study approval was given by Saveeetha scientific research committee with ethical number IHEC/SDC/FACULTY/22/GPATH/440.

Patients with Type 2 diabetes mellitus with a blood sugar level of more than 200 mg/dl. The age of patients over 40 years, who were diagnosed as OSCC and admitted for excisional biopsy, were included in this study. Patients with other oral lesions and insufficient cancer tissues were excluded from the study. Groupings were subdivided as group 1 control (n=5) , group 2 precancerous (n=5), group 3 carcinoma tissue (n=5).

Isolation of miRNA

miRNA was isolated from control and experimental samples using TRIR (total RNA isolation reagent) kit. 1 ml TRIR is used to homogenize 100 mg fresh tissue. The homogenate was immediately transferred to a microcentrifuge and kept at -80°C for 60 min to completely dissociate the nucleoprotein complexes. Then, 0.2 ml of chloroform was added, vortexed for 1minutes and placed on ice at 4°C for 5 minutes. The centrifugation of homogenates is performed at 12,000 x g for 15 min. Then, the aqueous phase was transferred to a fresh microcentrifuge tube. Then an equal volume of isopropanol was added, vortexed for 15 sec and placed on ice at 4°C for 10 min. The samples were centrifuged at 12,000 x g for 10 min at 4°C. A new microfuge tube was used to properly

transfer the aqueous phase. The RNA pellet was washed with 1 ml of 75% ethanol by vortexing and then centrifuging was performed for 5 minutes at 7,500 x g (4°C) later the supernatant was discarded. The supernatant was removed, and the RNA pellets were combined with 50µl of Milli-Q water that had been autoclaved before being heated in a water bath for 10 minutes at 60°C to dissolve them.

Quantification of RNA

The absorbance (A) at 260/280 nm was measured in order to spectrophotometrically quantify the diluted RNA sample. One absorbance at 260 nm is produced by 40 ng of RNA in 1 ml. Therefore, by multiplying A260 of the sample by 40 and the dilution factor, the amount of RNA in the sample may be calculated. The proportion between an RNA preparation's absorbance at 260 and 280 nm can be used to determine its purity. Good quality RNA is typically defined as having an absorbance ratio of >1.8 at 260/280 nm (Fourney et al., 1988). The recovered RNA was 1.8 percent pure.

Reverse Transcriptase – Polymerase Chain Reaction (RT – PCR)

RT-PCR is an approach for converting and amplifying a single stranded RNA template to yield abundant double stranded DNA products.

1. Initial strand reaction: Reverse transcriptase, Oligo dT, and dNTPs are used to create complementary DNA (cDNA) from the mRNA template.
2. Second strand reaction: Following the completion of the reverse transcriptase reaction, the "second strand reaction"—a type of conventional PCR—is started.

Principle

A technique for amplifying cDNA copies of RNA is called RT-PCR. It is the enzymatic transformation of one cDNA template from one mRNA template. An RNAdependent DNA polymerase extended a specific oligodeoxynucleotide primer that had previously hybridized to the mRNA to produce a copy of the cDNA. For DNA synthesis on the first strand we bought the RT kit from Eurogentec (Seraing, Belgium).

Reagents

1. 10X RT buffer: 1.4 ml of 10X RT buffer are available in one vial.
2. EuroScript reverse transcriptase: One tube containing 75 µl of Moloney
Murine leukemia virus reverse transcriptase (3750 U at 50 U/µl).
3. RNase inhibitor: One tube containing 120 µl of RNase inhibitor (2400 U at 20 U/µl).
4. 2.5 mM dNTP Mix: One tube (1.25 ml) of dATP, dCTP, dGTP and dTTP in autoclaved, deionized water titrated with NaOH to pH 7.0.
5. 25 mM MgCl2: One tube (1.5 ml) of 25 mM MgCl2.
6. Oligo d(T): One tube (50 µl) containing 50 µM oligodeoxynucleotides of sequence d(T) in 10 nM Tris-HCl, pH 8.3.
7. RNase free water: One tube (1.75 ml) of DEPC water was mixed gently and spun briefly, kept in the thermocycler. Initiation step at 25°C for 10 min, Reverse transcriptase step at 48°C for 30 min, inactivation of RT enzyme step at 95°C for 5 min. After the reaction, samples were stored at -20ºC or proceeded to the PCR.

Quantitative Real Time PCR

Principle

A gene is intended to be made in a PCR (Polymerase Chain Reaction) in large numbers of copies. A PCR involves three main processes, which are as follows: Denaturation for 3 minutes at 94 °C: All enzymatic processes halt during the two to five minutes of denaturation at 94 °C during which the double strand of DNA melts open to form a single strand. 30 seconds of annealing at 54–65 °C: To ensure the extension process, ionic bonds are continuously established and destroyed between the primer and the single-stranded template. Extension at 72°C for 30 seconds: Primers on sites where there is no exact match become loose once more (because to the higher temperature) and do not extend the fragment.The polymerase adds dNTPs from 5' to 3', reading the template from 3' to 5' side; bases are added complimentary to the template. These bases are attached to the primer on the 3' side. Since both strands are copied, the number of copies of the gene increases exponentially during PCR .

Reagents

1. 2X Reaction buffer: The PCR master mix kit was purchased from Takara Bio Inc., Japan. Contains TaKaRa Ex Taq HS (a hot start PCR enzyme) dNTP Mixture, Mg2+, Tli RNase H (a heat-resistant RNase H that minimizes PCR inhibition by residual mRNA), and SYBR Green I.
2. Forward primer (10µM)
3. Reverse primer (10µM)
cDNA- Template
5. Autoclaved milli Q water
6. Primers: The following gene specific oligonucleotide primers were used.

Procedure

Real Time PCR was carried out on the CFX 96 Real Time system (Bio-Rad). The reaction mix (10 µl) was prepared by adding 5 µl of 2X reaction buffer, 0.1 µl of sense and antisense primer (for PEPCK, G-6-Pase, β-actin), 1 µl of cDNA and 3.8 µl of sterile water. The thermal cycler protocol was as follows: Initial denaturation at 95˚C for 3 min, followed by 40 cycles of PCR, denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 20 sec. All reactions were performed in triplicate

along with no template control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 50-95˚C for each sample to determine the presence of multiple amplicons, non-specific products and contaminants. The results were analyzed using CFX 96 Real Time system software (Bio-Rad). As an invariant control, the present study used rat β-actin.

Human GLUT 1 gene specific primers
FW-5'-TGTGCAACCCATGAGCTAA-3'
RW-RW-5' CCTGGTCTCATCTGGATTC-3'

3. Results

miR23a association with GLUT1 in Oral squamous cell carcinoma were analyzed through Quantitative real time Polymerase Chain reaction were the interpretation reveals increase in fold change of miR23a (figure 1) in Cancer tissue compared with the control and precancerous samples.In RT-PCR. The amplification of miR23a is increased between 30 to 40 cycles above the threshold level in relation between 280 to 300 RFU (Figure 2). There is increase in fold change of GLUT 1 (figure 4) in Cancer tissue compared with the control and precancerous samples. In RT-PCR The amplification of GLUT is increased between 20-30 cycles above the threshold level in relation between 300 to 320 RFU in RT-PCR (Figure 5).

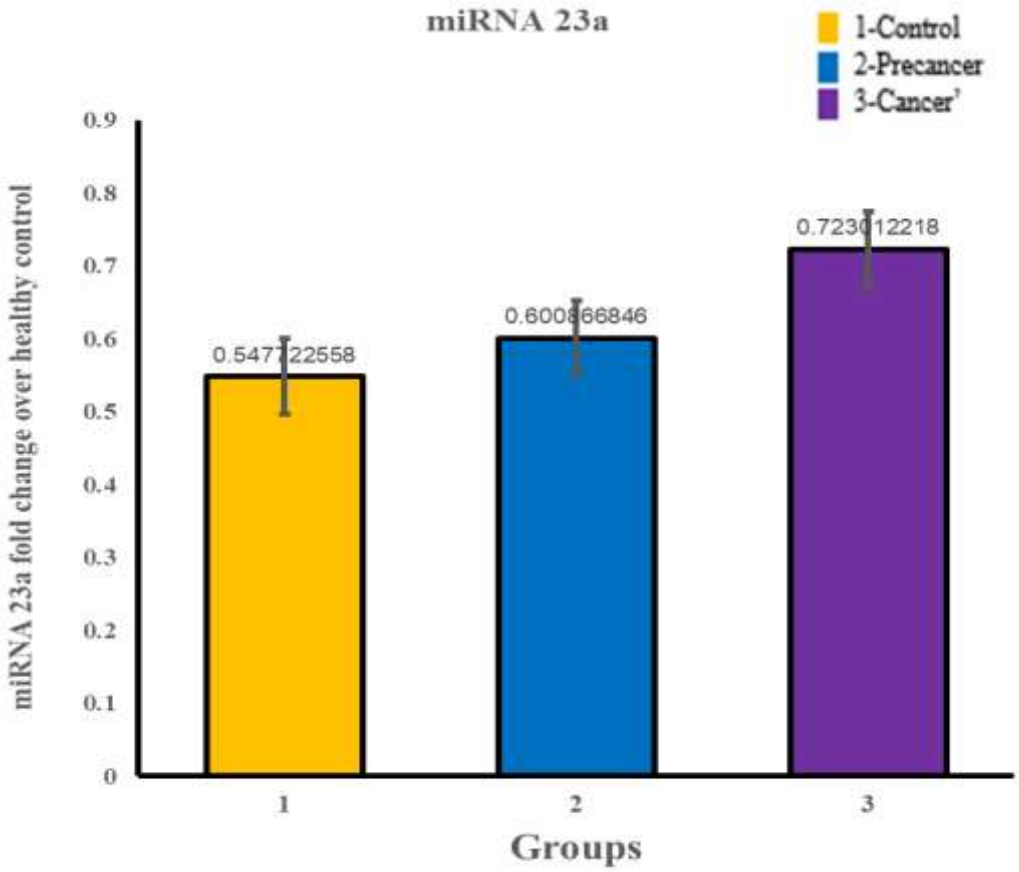

Figure 1 : X -axis represents study groups . The Y-axis depicts the miR23a fold change over healthy control. Cancer tissue showed increase in fold change compared to precancerous and control tissue

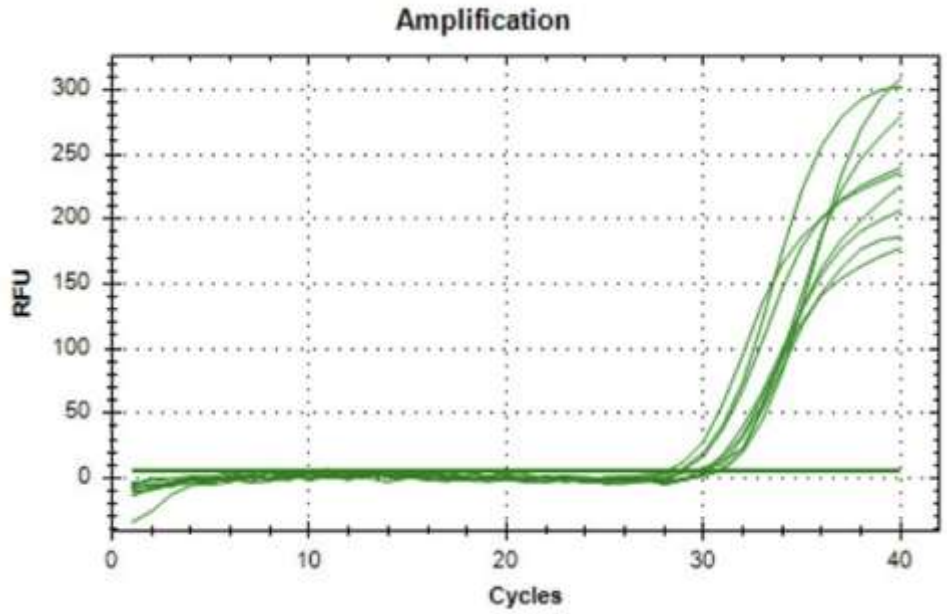

Figure 2 : X axis shows the number of cycles in RT-PCR and Y axis shows Relative fluorescence unit (RFU).

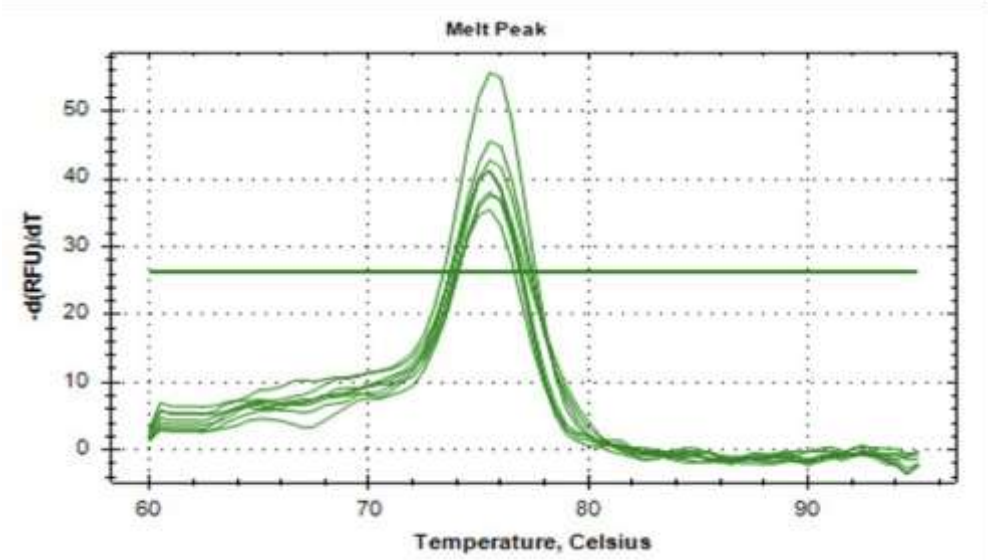

Figure 3 : Above graph depicts the melt peak of quantitative PCR results. The X- axis shows the temperature in celsius , Y-axis shows the differentiation of the Relative fluorescence unit (RFU).

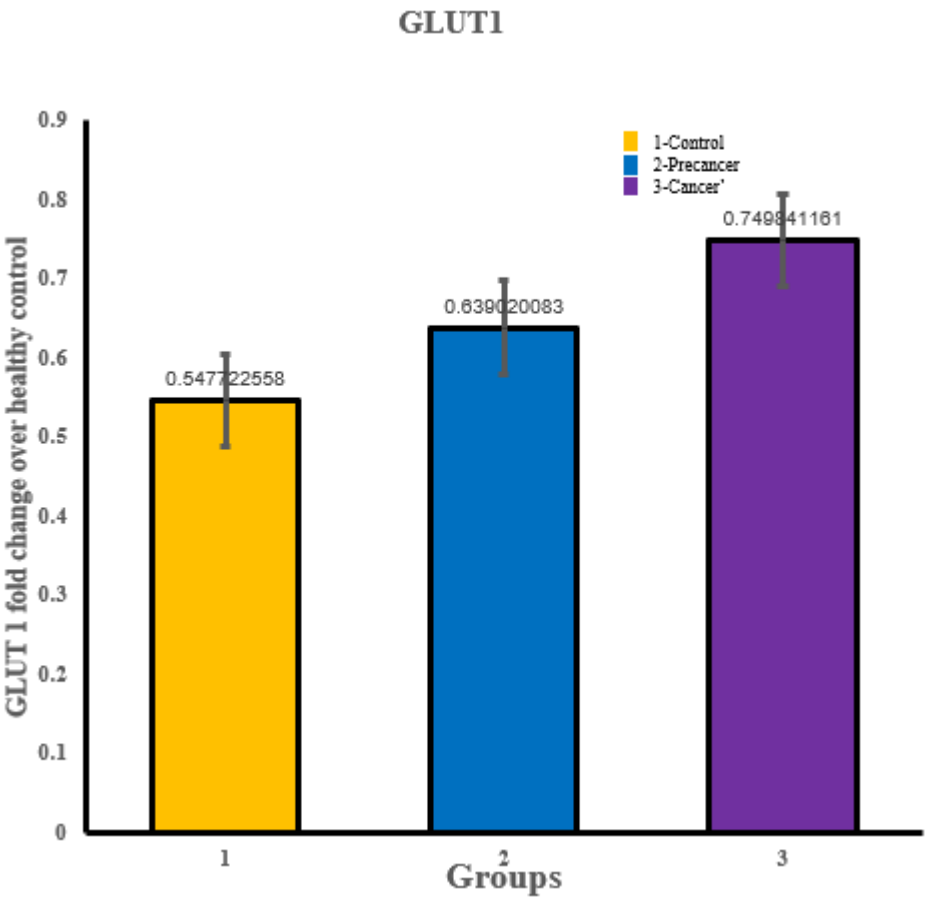

Figure 4: X -axis represents study groups . The Y-axis depicts the GLUT 1 fold change over healthy control. Cancer tissue showed increase in fold change compared to precancerous and control tissue

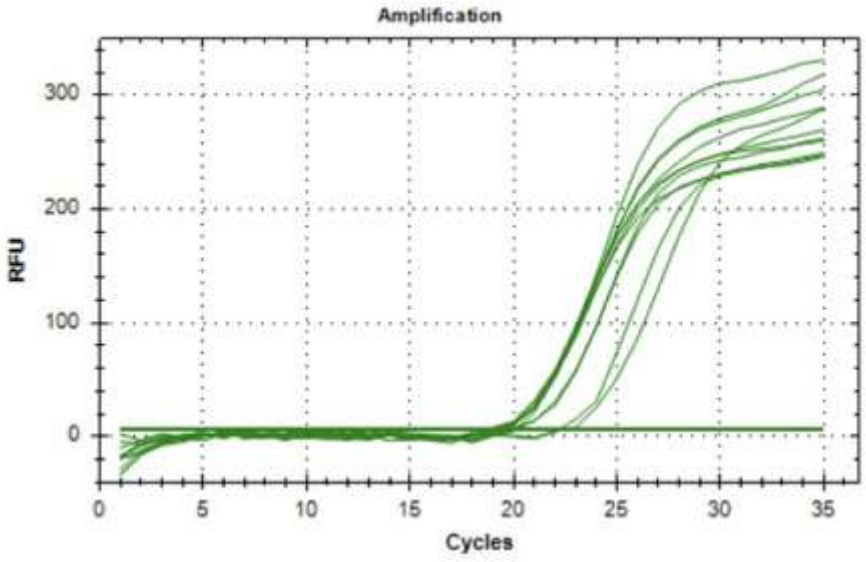

Figure 5: Above graph amplification cycle of RT PCR in GLUT 1. The X axis shows the number of cycles in RT-PCR and the Y axis shows the Relative fluorescence unit (RFU).

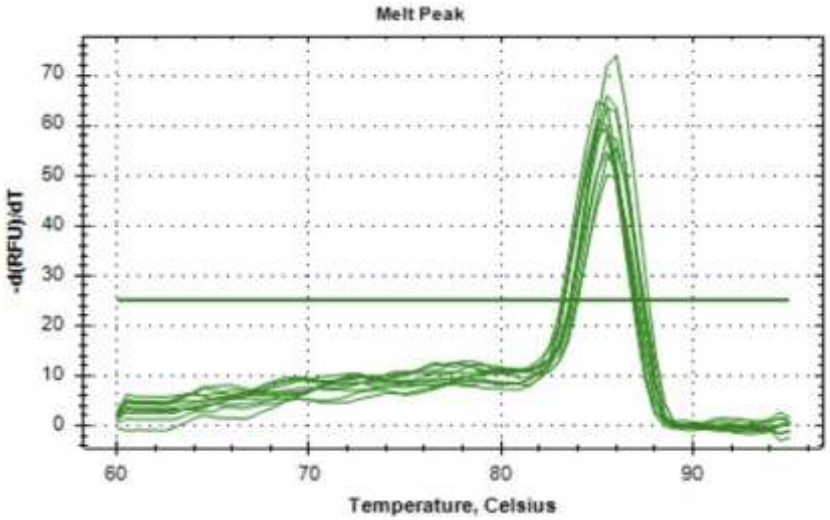

Figure 6: Above graph depicts the melt peak of quantitative PCR results. The X- axis shows the temperature in celsius , Y-axis shows the differentiation of the relative fluorescence unit (RFU).

The results showed that there is a significant increase in the fold change of diabetic cancer tissue miR23a over healthy control (Figure 1). There is evident through the quantitative PCR which shows amplification cycle was exponentially rising relative to 30 to 40 cycles with DNA formation at 320 Relative fluorescence unit (RFU) from the threshold level (Figure 2) and Figure 3 shows denaturation of proteins in miR23a with a melt peak analysis by binding of samples with SYBR green dye. Similarly, there is a significant increase in the diabetic cancer tissue GLUT1 level in the fold change over the control (Figure 4) and is evident through the quantitative PCR at 30 to 35 cycles amplification cycle with DNA formation at 330 Relative fluorescence unit (RFU) from the threshold level (Figure 5) and Figure 6 indicates the peak with denaturation of proteins in GLUT 1 analysis by binding of samples with SYBR green dye. With the above observed results we can come to a conclusion that an increase in miR23a fold change accelerates GLUT1 in Diabetic OSCC patients which promotes cancer cell growth.

4. Discussion

The present study demonstrated that miR-23a was involved in Oral squamous cell carcinoma (OSCC) revealing it to be an oncogenic miRNA. miR-23a up-regulates GLUT-1 thereby accelerates glucose uptake which results in the proliferation of OSCC cells. miR-23a have been extensively expressed especially in hepatocellular cancer (25). Its biological functions are spread over carcinogenesis, metastasis, cancer progression and drug resistance. In previous reports it has been suggested that it would have potential as an rising targetable entity in most cancers treatment. In this study, GLUT1 is upregulated by miR-23a, which is evident from the quantitative PCR, thereby accelerating the glucose metabolism. This increased glucose metabolism facilitates the abnormal proliferation of tumor cells. In a previous study, researchers have concluded that miR-23a could be used as a screening test for early detection of hepatocellular carcinoma and that it is a potential prognostic biomarker as it is associated with tumor size, and multiple focal liver lesions (26). Rabih et al., concluded that miRNA genes have been classified as either oncogenes or tumor suppressor genes in which specifically miRNA-23a misexpression contributes to many diseases, including cancer (27).

The most prevalent kind of oral cancer was OSCC which has a 5-year overall survival rate between 40% and 50%. Chen-Xi Li et al., evaluated in his study that GLUT-1 overexpression was in connection with aggressive clinical features and shortened overall survival in OSCC (28). Ji Wang et al., revealed in their study, which is in accordance with the current study, that the expression status of GLUT1 is a vital prognostic indicator and promising therapeutic target in solid tumors. (29)

Martin Kunkhel et al., proved that both glucose transport and glucose metabolism determine the glycolytic tumor phenotype, which is a significant negative biomarker of prognosis and overall survival in patients with OSCC (30). This is in contrast to the present study which reveals that GLUT1 can be a better therapeutic target for the patients with OSCC.

Shibuya et al., revealed in their study that GLUT-1 dependent glucose metabolism has a pivotal role not only in the growth and survival of cancer stem cells (CSC) but also in the maintenance of their stemness (31). They also suggested GLUT1 as a promising target for cancer stem cell directed therapy which is in line with the present study. Results from the study by A W Eckert et al., suggested that GLUT-1

expression is an independent prognostic marker for routine assessment of OSCC. Cancer cells derive energy for cell division and proliferation from the glucose metabolism which is promoted by the overexpression of GLUT1 (32). Therefore, the treatment for various types of cancer is inhibition of GLUT1, which serves as a potential therapeutic target. In 2022, Jing Gao et al., indicated in their study that miR-23a-3p can inhibit the PI3K/Akt signaling pathway by targeting RUNX2 and inhibit the malignant evolution of oral cancer (33). Consistent with previous research, the current study showed that GLUT1 expression levels were considerably higher in tumor tissues than in underlying healthy tissues in OSCC patients.

Yuan‑Hua Chen et al., proved in their study that according to invitro cell tests, GLUT1 knockdown by siRNA decreased cancer cell proliferation and glucose uptake, while GLUT1 overexpression enhanced cancer cell proliferation and glucose uptake. These findings further suggested that GLUT1 plays an oncogenic function in oral cancer which is in light with the present study (34). In 2021, Xue Wang et al., concluded in their study that through downregulation of miR-23a, the recently discovered lncRNA ZEB1-AS1 acts as a tumor promoter in OSCC. These findings suggest that ZEB1-AS1 may be a promising molecular target for the treatment of oral cancer (35). This is found contradicting the present study that there is overexpression of miRNA-23a present in OSCC cancer tissues.

The in vitro experiments revealed that overexpression of miRNA-23a could significantly mediate the upregulation of GLUT1, while overexpression of GLUT1 did not significantly alter the expression of miRNA-23a (36). As a result, miRNA-23a may function as an upstream activator of GLUT1 in OSCC, promoting tumor cell proliferation and glucose uptake. According to earlier research, miRNAs control the expression of GLUT1, which contributes to the growth, development, and progression of cancer. In the present study, a novel miRNA regulator of GLUT1 has been identified in cancer biology.

The main limitation of the study is that the sample size taken is small. Therefore for further correlation analysis studies with a larger sample size is required. Moreover, there is significant correlation between miRNA-23a and GLUT1 expression in cancerous and healthy tissue (37). Therefore, miRNA-23a regulates GLUT1 directly and the interaction between miRNA-23a and GLUT1 may be mediated by specific pathological factors such as tumor suppressor or oncogenic signaling pathways. As a result, miRNA-23a was identified for overexpression in OSCC. miRNA-23a promotes tumor cell differentiation and glucose metabolism in OSCC by acting as an upstream activator of GLUT1.

5. Conclusion

From this study we can conclude that miRNA-23a can promote cancer cell proliferation in Oral squamous cell carcinoma (OSCC), by functioning as an upstream activator of GLUT1 and promoting glucose metabolism. Further studies on potential therapeutic targets for the treatment of different types of cancer, especially squamous cell carcinoma can be done by targeting miRNA23a expression with a subsequent GLUT 1 inhibition.

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Conflict of interest

All the authors declare that there was no conflict of interest in the present study.

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