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CANCER CHEMOPREVENTION BY METFORMIN HYDROCHLORIDE COMPARED TO PLACEBO IN ORAL POTENTIALLY MALIGNANT LESIONS: A RANDOMIZED CLINICAL TRIAL

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ABSTRACT

Background: The aim of the present randomized controlled clinical trial was to provide further molecular evidence for the postulated cancer chemopreventive potential of systemic Metformin hydrochloride on oral potentially malignant disorders (OPMDs), shown by simultaneous work by our team.

Subjects and methods: Forty non diabetic patients, suffering from oral potentially malignant lesions, namely leukoplakia and oral lichen planus, were retrieved from the outpatient clinic of Oral Medicine and Oral Diagnosis Department and the Diagnostic Center, Faculty of Dentistry, Cairo University, Egypt. The trial was conducted with two arms parallel groups, with each group consisting of 20 patients. One group received Metformin hydrochloride at a single daily dose of 500 mg, the other received a placebo. Recommended standard health and dental care was given to both groups and risk factors were eliminated. Clinical data, toluidine blue test, biopsies, immunohistochemical staining with cyclin-A2, tissue and salivary miRNA-31 and miRNA-210 levels evaluation, were all performed at baseline and at 3 months point.

Results: there was significant decrease of the lesion size in Metformin group while there was significant increase of the lesion size in placebo group (P-value < 0.05).

When compared to placebo, Metformin proved to affect the cell cycle-dependent mechanisms and epigenetic factors that could affect the progression of oral potentially malignant lesions, particularly with its ability to decrease Cyclin-A2 expression (P-value= 0.016), to down-regulate miRNA-31 and miRNA 210 levels (in both tissues and saliva). Changes in toluidine blue staining intensity, however, was not statistically significant.

Conclusions: Our results supported the other clinical trial by our team, showing that Metformin can offer a cancer chemopreventive effect in OPMDs, as it was capable of reducing significantly the size of the lesions and the degree of epithelial dysplasia together with down expression of Cyclin A-2 and cancer associated miRNA-31 and miRNA-210, which gave additional molecular validity for the concomitant results.

Keywords: Chemoprevention, metformin, oral potentially malignant lesions, oral cancer, oral squamous cell carcinoma, OPMDs, miRNA-31 miRNA -210, Cyclin A2, anticancer effect.

INTRODUCTION

In 2005, The WHO categorized any lesion or condition that carries the risk of malignant transformation as an "oral potentially malignant disorder" (OPMD) **Al-Hashimi et al.** (2007)**.** OPMDs include leukoplakia, erythroplakia, oral submucous fibrosis, oral lichen

planus, oral lesions of lupus erythematosus, actinic cheilitis and other miscellaneous lesions **Napier & Speight** (2008)**.** The process of malignant transformation of OPMDs is a complex multistep process associated with several successive mutations in oncogenes, tumor suppressor genes together with many other genes resulting in sustained cellular proliferation, evasion of cell suppressors and cell death, stimulation of angiogenesis, invasion and metastasis **Rivera et al.** (2017) .Oral cancer development is associated with numerous molecular alterations in the essential genes that regulate critical cellular processes, such as cell cycle, cell proliferation and apoptosis **Zhong et al. (2017).**

Traditionally, *oral epithelial dysplasia* was regarded as the progenitor for malignant changes. Therefore, there has always been a challenge for pathologists to assess the degree of dysplasia in oral potentially malignant disorders with accuracy, for better prognosis prediction and management **Kujan et al.** (2006). In the update of WHO classification **El-Naggar et al.** (2017), a **binary system** originally supposed by **Kujan et al.** (2006). was adopted namely consisting of low grade and high-grade dysplasia. Low risk oral leukoplakia comprises: no dysplasia and mild dysplasia and high-risk oral leukoplakia includes moderate and severe dysplasia. The cut-off point between low grade dysplasia, as originally suggested by **Kujan et al.** (2006) are 4 architectural and 5 cytological changes irrespective of the level within the epithelium. The low-grade lesions in the binary system have less than 4 architectural or less than 5 cytological abnormalities.

Although it is established that OPMDs are statistically more likely to become malignant, it is not necessary that every lesion will progress to cancer. Although the clinical parameters described earlier may help in clinical risk stratification, the gold standard diagnostic procedure remains biopsy and histologic examination **Macey et al.** (2015). The ultimate goal of *oral epithelial dysplasia* diagnosis and grading is to provide patients with the best management and care. As it stands, the grade of *oral epithelial dysplasia* is still the most important prognostic factor for malignant transformation. However, even in the case of severe epithelial dysplasia, studies have shown that the malignant transformation rate varies considerably, from 3% to 50% and appears to be dependent on study design and population characteristics **P Holmstrup et al.** (2006).

The cornerstone in terms of risk assessment is to discover a biomarker that can be used in a histologic or chairside test to predict malignant transformation of oral lesions. There have been many studies investigating various potential markers, but to date, no single biomarker has proved to be of clinical value **Speight et al.** (2018).

OSCC arises from the accumulation of successive genetic and epigenetic alterations in a multistep process over many years, transforming a single cell or a clone of cells of the oral mucosa into a malignant tumor. Specific key genes and proteins are affected in this process as they function in several signaling pathways related to cell proliferation, regulation, and differentiation, ultimately leading to tumor growth and progression **Khan & Bisen** (2013).

Deregulation of the cell cycle mechanism is a critical event in carcinogenesis and it is emerging as a central theme in oral carcinogenesis. **Mishra** (2013). **Monteiro et al.** (2018) reported that cyclins were highly expressed in a cohort of OSCC . High expression values for cyclin A2 were observed in more than 80% of tumor cells, and lower frequencies for cyclin B1 and E1. These levels of expression reflect the relevant role of these proteins in OSCC pathogenesis **Chen et al.** (2003); **Saarilahti et al.** (2003) ; **Fraczek et al**. (2008). In addition, **Mishra et al.** (2013) observed a significant association between high cyclin A2 expression and advanced stage tumor, advanced tumor size (T), and presence of nodal metastasis.

Recently, epigenetic alterations, including promoter/intragenic methylation and miRNAs deregulation, have been linked to the development of oral cancers **Irimie et al.** (2018).

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Small RNAs are defined by their length (20–30 nucleotides) and are classified into three classes in animals: **miRNA (miRNA)**, **short interfering RNA siRNA** and **PIWI-interacting RNA (piRNA)**. miRNAs constitute a dominating class of small RNAs in most somatic tissues and are produced by two RNase III proteins, Drosha and Dicer **Ghildiyal & Zamore** (2009).The recent discovery of hundreds of miRNAs, from various organisms and functional assays have determined that miRNAs assist important functions in cell growth, differentiation, apoptosis, stress response, immune response, and glucose secretion **Mudduluru et al.** (2011)

Deregulation of certain miRNAs has been identified in cell experiments, clinical specimens, saliva or plasma samples of patients with oral malignancies. The transcriptome analysis, or the detection of these (miRNAs), can function as an accurate marker of cancer. The most reported possible players in the tumorigenesis of OSCC are miRNA-21, miRNA-31, miRNA-221, miRNA- 184, miRNA-133a, miRNA-375, and let-7b **Gorenchtein et al.** (2012). Actually, plasma and salivary miRNA-31 was considered a likely early marker of OSCC **Liu et al.** (2010); **Hung et al.** (2014); **Chung-Ji Liu et al.** (2012). The study of **Jamali et al.** (2015) reported that miR-210-3p expression is elevated in Prostate cancer (PCs) tissues compared with the adjacent prostate epithelial tissues. Interestingly, the expression levels of miR-210-3p increases steadily from non-bone metastatic PCa tissues, bone metastatic PCa tissues to metastatic bone tissues and high expression of miR-210-3p positively correlates with the clinicopathological characteristics and bone metastasis status of PCa patients. Furthermore, upregulating miR-210-3p enhances, while silencing miR-210-3p suppresses invasion and migration of PCa cells in vitro. Importantly, silencing miR-210-3p significantly inhibits bone metastasis of PC-3 cells in vivo.

The management of OPMDs is highly variable according to the type of the lesion and practicing clinicians. However, various treatment modalities exist, such as: local therapies, systemic therapies and surgical removal. Local therapies include laser therapy, photodynamic therapy, cryotherapy and conventional surgical removal but no treatment so far has gained universal acceptance **Lodi et al.** (2016). The systemic therapies include Retinoids, extracts of green tea, Cyclooxygenase-2 inhibitors, epidermal growth factor inhibitors, peroxisome proliferator- activated receptor C agonists and many other pharmacological agents **Hansen et al.** (2000).

The reason why surgical removal may not always be beneficial, is that the visible clinical lesions may be surrounded by genetically altered, cancer stigmatized epithelial cells that can't be detected by routine clinical or histopathological examination. This concept is referred to as "field cancerization". A possible result of such phenomenon is the lack of resection of all affected tissue with recurrence and development of carcinoma from residual genetically altered cells **Hong & Sporn (1997).**

No single local or systemic therapy has shown to reduce the recurrence rates or decrease the malignant development in long-term follow-up studies. It is well established that "no treatment" results in malignant development at an annual rate of 2–3%. In the complex management of OPMDs, the overall purpose of treatment is to reduce this percentage. The lack of randomized clinical trials favoring a specific agent, raises concerns on finding alternative scenarios **S. Warnakulasuriya et al**. (2007)

Consequently, chemoprevention is thought to be the most effective strategy when treating patients with OPMDs, aiming at improving the survival rates and preventing the progress of these lesions into oral squamous cell carcinomas (OSCC) **Costea et al**. (2018). Several cancer chemopreventive agents have been used, such as: retinols, beta carotene, curcumin, and others. However, the lack of clinical efficiency, associated toxicities and relapse following cessation of therapy, have made the role of these agents limited **Maher et al.** (1997) and **Saman Warnakulasuriya et al.** (2020)

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Metformin HCL (1,1-dimethylbiguanide hydrochloride) is a biguanide oral hypoglycemic drug that is widely used in the treatment of type 2 diabetes mellitus, polycystic ovarian syndrome and diabetes prevention **Krentz & Bailey** (2005). Metformin, which is one of the most widely prescribed anti-diabetic drugs, has recently received attention because of its potential anti-cancer effects that are thought to be independent of its hypoglycemic effects **Evans et al.** (2005). Moreover, systemic Metformin hydrochloride tablets were associated with improved survival rates among diabetic patients with head and neck cancers **Rêgo et al.** (2017).

The first observation that Metformin reduced the risk of cancer was made in a population-based case control study of patients with type 2 diabetes (T2D) **Evans et al.** (2005). The interest in Metformin for cancer prevention and treatment is based on clinical studies that show that the use of Metformin is associated with significantly lower cancer incidence in diabetic patients, although untreated T2D is associated with an increased cancer risk, attributed mostly to the growth promoting effect of chronically elevated plasma glucose and insulin levels **Huang et al.** (2008). Several clinical trials using Metformin as a treatment in non-diabetic cancer patients have produced encouraging results **Evans et al.** (2005); **Kalogirou et al**. (2016); **Kato et al**. (2012). To date, Metformin has been shown to reduce the development of lung, hepatic carcinoma, breast, and colorectal cancers. A number of studies have been published on the mechanisms involved in the antitumor effects of Metformin. However, Metformin mediated anti-neoplastic effects and the underlying mechanisms have not been fully elucidated **Daugan et al.** (2016).

Several lines of evidence have suggested that Metformin regulates DNA methylation in cancer cells through Tricarboxylic acid (TCA) cycle intermediate metabolites **Cuyàs et al.** (2018), as well as histone acetylation **White-Al Habeeb et al.** (2016). A novel finding from a recent study was that Metformin suppressed melanoma progression by affecting epigenetic mechanisms **Tseng et al.** (2019). For instance, **Pulito et al.** (2017) demonstrated that Metformin exerted anti-neoplastic activity through modulating the Dicer processing enzyme, which was accompanied by changes in a subset of miRNAs, identifying a new role of Metformin in regulating the progression of cancer. Interestingly, **Wu et al.** (2019) found that Metformin represses the expression of Head and Neck Squamous Cell Carcinoma (HNSCC) stem cell programs, and causes the loss of expression of markers associated with cancer stem cells and promotes HNSCC terminal differentiation. It has been suggested that the promising anti-neoplastic effects of Metformin would be useful in preventing the malignant transformation of oral potentially malignant disorders **Chiang et al.** (2017).

We hypothesized that if a chemotherapeutic agent as Metformin could affect a critical miRNA (as miRNA-31) or one of the cyclins (cyclin A2), this would halt the progression of malignant transformation and would explain its basic mechanism of action, if it proved clinically and/or histopathologically to have any.

Consequently, this research was conducted to assess the clinical, histopathological and immunohistochemical expression profiles of cyclin A2, and miRNA-31 following systemic administration of Metformin tablets in patients with oral potentially malignant disorders compared to placebo, in order to provide further supportof its efficacy to prevent or at least reduce the incidence of malignant transformation.

SUBJECTS AND METHODS

The current study is registered on ClinicalTrials.gov (NCT03684707)

Trial Design

The current study is a randomized controlled clinical trial with two arms parallel groups. The study was triple-blinded trial where participants, clinicians and outcome assessors were

blinded. It is a superiority trial, where Metformin hydrochloride was supposed to be superior to placebo regarding oral carcinogenesis chemoprevention. The patients were randomized by Excel software and allocation concealment was done by the help of another operator in the same department. The allocation ratio was 1:1.

Patient Selection

Patients were selected from the outpatient clinic of Oral Medicine and Oral Diagnosis Department, and the outpatient diagnostic center, Faculty of Dentistry, Cairo University, Egypt, from October 2018 to January 2020. A total of 40 patients with OPMDs (oral lichen planus, leukoplakia) were recruited. Full medical history was obtained using the Cornell Medical Index **Abramson** (1966). HbA1C was measured to exclude undiagnosed diabetic patients. These were the same patients mentioned in the concomitant trial **Abdel-Azim et al. (2021).**

Inclusion criteria

- Both genders with age range20-70 years were included in the present study.
- All included patients were confirmed cases with oral potentially malignant lesions including atrophic oral lichen planus and leukoplakia.

Exclusion criteria

- Diabetic patients
- Patients suffering from cardiovascular, lung, renal or liver diseases
- Patients on H2 blocker & proton pump inhibitors therapy as Ranitidine
- Patients with allergy or sensitivity to Metformin therapy
- Systemic and/or local drug therapy within the last 3 months.
- Patients on steroidal or non-steroidal anti-inflammatory drugs (NSAIDs) for at least the last 6 months.
- Patients on Retinoid, green tea supplements
- Patients with already diagnosed malignant lesion/lesions
- Pregnant and lactating females
- Vulnerable groups as prisoners, mentally disabled, etc.
- Patients who cannot be committed to the follow up appointments.

Ethical Issues

The protocol was reviewed by the research ethics committee and accepted - Approval code: (18- 9-51) at Faculty of Dentistry, Cairo University and was conducted according to the declaration of Helsinki and all patients understood the nature of the study and signed the informed consent.

INTERVENTIONS

1-Initial Intervention

The initial therapy was applied to all patients at baseline and included patient education and removal of any predisposing factors. The most suspected areas were biopsied with the aid of toluidine blue stain. Part of the biopsy was used for quantification of mi-RNA31 and miRNA 210 . The other part was formalin fixed and paraffin embedded. Histological sections were stained with H&E for diagnosis and grading of dysplasia and immunohistochemistry was then carried out for cyclin A2.

Metformin was prescribed to the study group (Group I) in the form of 500 mg systemic Metformin hydrochloride once per day (Glucophage HCL 500 mg tablets) for 3 months. The

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control group (Group II) received the same form of the tablet without active ingredient (placebo) once daily, for 3 months.

2- Maintenance Phase

For the 3 months study period, detailed follow up of the patients was performed. Patients were evaluated in biweekly recall visits for reinforcement. During the follow up appointments, careful visual inspection and palpation under proper illumination to detect any clinical signs of malignant transformation (erythroplasia, induration, ulceration, bleeding……etc.) were done. Lymph node examination for all cervicofacial lymph nodes to detect any abnormality was performed as well and toluidine blue staining for the lesions at every follow up visit was carried out.

Three different pathologists examined each case blindly for the diagnosis and grading of the degree of epithelial dysplasia according to WHO Classification (2017) (**El-Naggar et al., 2017).** All lesions were captured using Canon camera 650D model with automated settings. The images were saved in tiff format (as a lossless format). The lesion size was measured by assessing the surface area (SA) by the ImageJ software at baseline and after 3 months. Routine H&E sections and immunohistochemistry were visualized using a Zeiss microscope and images were captured.

Tissues were mounted on positively charged slides, prepared, stained and captured for immunohistochemical analysis. ImageJ software was used to assess levels of Cyclin A2 and nuclear cell counts were calculated semi-automatically. Tissue was prepared for RNA Extraction. Mi-RNAs were extracted from saliva using miRNeasy extraction kit (Qiagen, Valencia, CA, USA). Reverse transcription was then carried out and Real-Time Quantitative PCR was performed.

Sample Size Calculation:

The outcome used (1ry or 2ry): 1ry outcome was clinical and histopathological changes in numerical values and the 2ry outcome was assessment of microRNA-31 and miRNA 210 in tissue samples and saliva and immunohistochemical analysis of cyclin A2 in tissues in numerical values. Values used for outcome were mean, standard deviation and percentage of change in the lesion size.

There were two entries: Entry 1: Group 1, where Proportion I was 0.58 and Entry 2: Group2, where Proportion I was 0.182. Alpha level of significance was 0.05. Effect size used in calculation was RR= 3.18. Power of the study was 80%. Statistical test used: z test: proportions: difference between 2 independent proportions. The calculated sample size was 22 patients per group. Increased number for anticipated missing data was added as 20% to the calculated sample size giving 31 subjects per group.

Statistical analysis used paired t-test and Chi-square test. Data were statistically analyzed by Microsoft Excel ® 2016[1], Statistical Package for Social Science (SPSS)® Ver. 24[2]. and Minitab[3] ® statistical software Ver. 16. Throughout three months follow up period from baseline up to three months, percentage of change was calculated according to the following formula

[1] Microsoft Cooperation, USA.

[2] IBM Product, USA.

[3] Minitab LLC, USA.

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$$\text{Percent Change} = \frac{\text{New Value} - \text{Old Value}}{\text{Old Value}} \times 100\%$$

If the result is positive, it is an increase.
If the result is negative, it is a decrease.

Results:

The number of males enrolled in the study was slightly larger in each group as in the Metformin group, there were 11 males and 9 females, and in placebo groups, there were 12 males and 8 females. These differences proved to be statistically insignificant by the Chi-square test. The incidence of the two studied lesions (leukoplakia and lichen planus) showed a distinct male vs. females. Lichen planus showed the reverse, where there was a significantly higher incidence in females vs. males (Chi-square = 13.6, p-value < 0.01).

The mean age for the Metformin groups was 47.1 (range 28–71) and for the placebo group was 50.8 (range 33–70). There was no significant difference between groups regarding age.
Percentage of change in the lesion size was calculated according to:

$$\text{Percent Change} = \frac{\text{New Value} - \text{Old Value}}{\text{Old Value}} \times 100\%$$

If the result is positive, it is an increase.
If the result is negative, it is a decrease.

The comparison between Metformin Hydrochloride group and placebo group is shown in table (1) and figure (1).

Regarding lesion size, mean percentage change ± standard deviation of Metformin and placebo group were (-11.61±16.06) and (0.85±17.2) respectively. Results of independent t test revealed that there was significant decrease of the lesion size in Metformin group while there was significant increase of the lesion size in placebo group (P-value < 0.05).
Regarding toluidine blue intensity, statistical analysis revealed insignificant decrease in both Metformin and placebo groups (P-value > 0.05).
Regarding Cyclin A2 expression, there was a significant decrease in Metformin group and a significant increase in placebo group (P-value < 0.05).
There were no statistically significant differences between placebo & metformin regarding the mean values of RNA 31 (either tissue or saliva) at baseline. On the other hand, the mean values of RNA 31 (either tissue or saliva) at 3 months for metformin was lower than that for placebo. That difference was statistically significant (P-value < 0.05).
Similar results were obtained with miRNA 210 tissue and salivary levels, where mean they showed a decrease in Metformin group at 3 months, significantly higher than the placebo group (P-value < 0.05).
Chi Square test results showed that there was significant difference between Metformin and Placebo groups regarding level of dysplasia (P-value < 0.05), as shown in table (2) and figure (2).

Table (1): Percentage change in Metformin Hydrochloride group compared to placebo in Oral Potentially Malignant Lesions

	Groups										**P-value**
	Metformin					**Placebo**					
	M	**SD**	**Median**	**Min**	**Max**	**M**	**SD**	**Median**	**Min**	**Max**	
Surface Area	-11.61	16.06	-6.91	-40.41	18.59	.85	17.20	-.62	-26.60	44.97	**0.023***

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Toluidine Blue Intensity	-2.48	5.32	-.63	-14.74	6.85	-1.12	2.90	-.69	-8.97	3.51	**0.327**
Cyclin A2	-11.07	14.45	-13.57	-39.30	23.18	1.00	15.93	1.60	-30.65	29.10	**0.016***
MiRNA210 (Tissue)	-35.24	21.30	-33.75	-70.02	-4.08	-2.25	5.48	.00	-17.36	3.41	**0.000***
MiRNA210 (Saliva)	-21.45	19.13	-23.69	-59.13	5.92	-1.45	18.29	-7.69	-17.23	60.79	**0.002***
MiRNA31 (Tissue)	-49.04	25.08	-51.72	-81.84	-1.71	-2.40	4.51	.00	-15.08	.95	**0.000***
MiRNA31 (Saliva)	-40.24	19.53	-38.72	-77.88	-4.14	-4.22	7.24	.00	-23.12	2.51	**0.000***

M; Mean, SD; Standard Deviation, Min; Minimum, Max; Maximum, P; Probability Level
Significant Difference

Table (2): Level of Dysplasia:

		Metformin	**Placebo**
No Dysplasia	**Baseline**	11.25 %	10 %
	Three Months	11.25 %	10 %
Low-Grade	**Baseline**	8.75 %	12.5 %
	Three Months	13.75 %	15 %
High-Grade	**Baseline**	5 %	2.5 %
	Three Months	11.25 %	10 %
P-value	**0.007***		

Significant Different

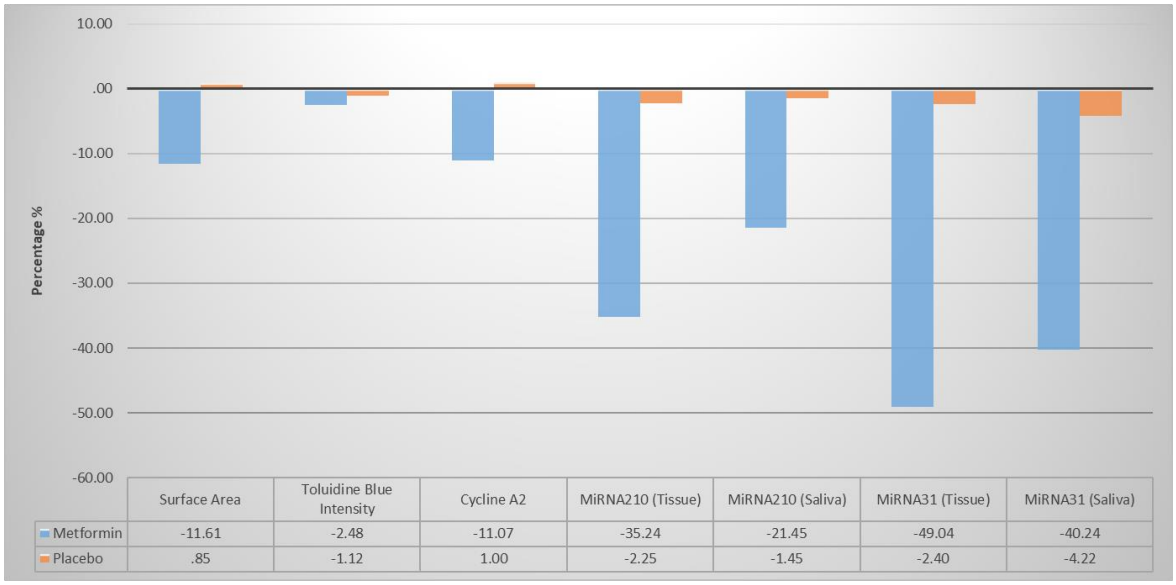

Figure (1): Bar chart revealing mean percentage change in studied items for Metformin Hydrochloride compared to placebo

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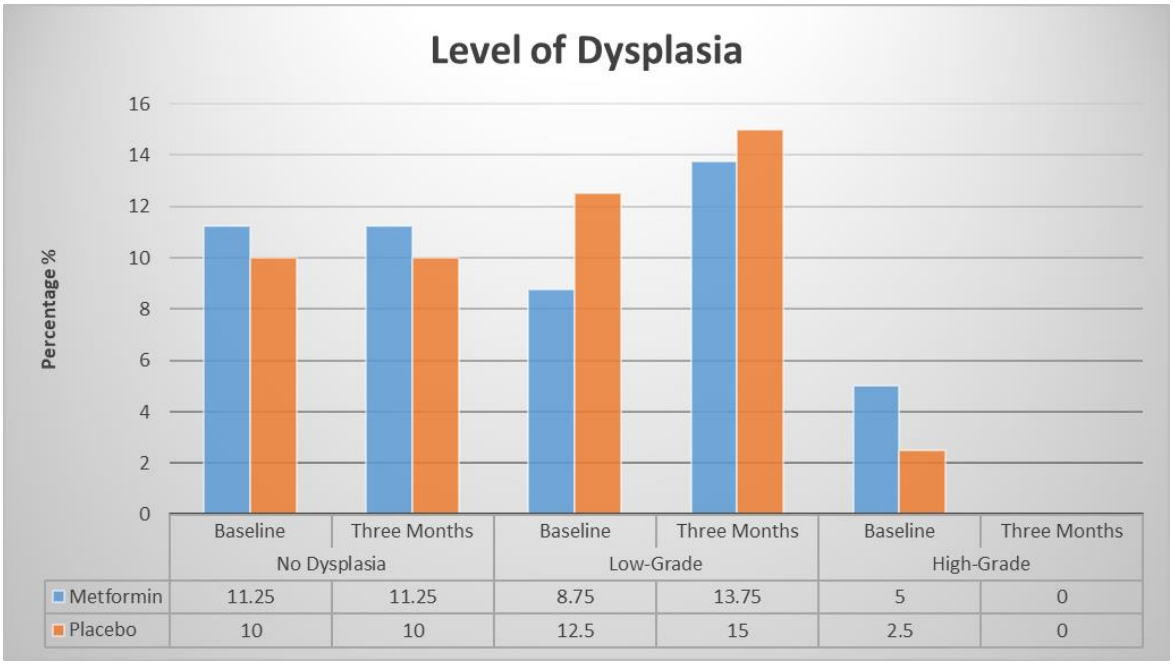

Figure (2): Bar Chart revealing level of dysplasia in studied groups

DISCUSSION

Although the normal oral mucosa can develop oral cancer, many oral squamous cell carcinomas (OSCCs) arise from potentially malignant diseases **Markopoulos et al. (2012) Saman Warnakulasuriya et al**. (2020)**.** Due to tumor invasion, cervical lymph node metastasis, and ultimate blood born dissemination, oral squamous cell carcinoma emerging from the oral mucosal epithelium remains a lethal and deforming disease. Each year, 300,000 new cases are seen worldwide, with a vital increase in incidence affecting young people in particular **Fitzpatrick et al. (2014).**

Theories of field cancerization suggest that there are cancer-induced modifications throughout both the oral cavity and upper aerodigestive tract. Actually, a particular subgroup of OPMD patients shows a high incidence of multiple lesions and multifocal tumor development. Detailed follow-up studies have shown that up to 24% of patients are affected by multiple site diseases **Mignogna et al.** (2007).

However, transforming normal stratified oral squamous mucosa into premalignant and subsequently, malignant tissue remains poorly understood. What is actually recognized is that it is a complex, multi-stage and multifactorial process in which the normal functioning of oncogenes and tumor suppressor genes is interrupted by accumulated genetic and epigenetic alterations. Cell cycle disruption is characterized by dysregulation, increased proliferation, and changes in differentiation, DNA repair, apoptosis, and cellular immunity in the earliest carcinogenesis phases **Tilakaratne et al**. (2019).

Palle Holmstrup et al. (2009) confirmed that the assumption of that removing the lesion would free the patients from the risk of cancer in the affected area was wrong and the issue is further complicated by the fact that 5–10% of leukoplakias contain carcinoma which was not initially noted on diagnostic biopsy but only afterwards on surgical excision.

According to field cancerization theory, chemoprevention drugs provide systemic control of the cancer transformation process compared to conventional surgical excisions that may be difficult to perform because their extent cannot be surgically managed. Also,

with surgical excision of OPMD lesions the subsequent risk of new-site OSCC development is not eliminated **Thomson et al.** (2017).

Metformin was considered to have the potential to be a candidate for such a strategy in the last ten or more years, given its low cost, favorable toxicity profile, and accumulating evidence regarding its anticancer efficacy **Vitale-Cross et al.** (2012).

Metformin is a first-in-line, widely used, inexpensive, and safe drug for the treatment of type 2 diabetes mellitus (T2DM). The hypothesis that Metformin has antineoplastic activity is supported by considerable laboratory evidence **Mignogna et al.** (2007). Many in vitro studies have shown direct antiproliferative actions of Metformin at millimolar concentrations **Kordes et al.** (2015), **Reni et al.** (2016) and **Zhao et al.** (2017) . Conflicting findings on Metformin's benefits in head and neck cancer care have been available in the clinical literature **Sikka et al.** (2012); **Becker et al.** (2014); **Lerner et al.** (2017) and **Rêgo et al**. (2017) .

Dysplastic and non-dysplastic lesions have been included in the current study because, even if the lesion is non-dysplastic, it has an abnormally hyperproliferative epithelium and may turn into a dysplastic lesion at any time. In that way, the usefulness of Metformin as chemopreventive agent would be to prevent the conversion of non-dysplastic lesions into dysplastic ones and to prevent the development of mild dysplasia into moderate and severe dysplasia, which can be transformed later into malignancy. In the present study, we didn't observe any dysplastic lesion reverting to no dysplasia although the degree of dysplasia did change its severity in high-grade lesions. At 3 months point, there was a significant regression of high- grade dysplasia in Metformin group compared to placebo.

As there is no agreement on the OPMD treatment and normally the protocol is based on a wait-and-see basis and follow up with eliminating profound risk factors such as smoking tobacco, alcohol abuse, etc. **Diajil et al.** (2013), thus, the administration of placebo besides the routine care protocol was considered totally feasible and ethical.

Eligible patients were randomly assigned (1:1) to receive oral Metformin (500 mg daily) or identical placebo tablets by a stratified computer-based randomization method. Also, all patients, doctors, and investigators were masked to drug allocation until the end of the trial. This was done to avoid selection bias.

The outcomes in the current study included a primary outcome, in the form of a clinical assessment of lesion progress. One of the clinical parameters taken was the percentage of change in the lesion surface area. The lesion size was selected because it indicates tumor cell proliferation and differentiation to show tumor progression or regression. It also indicates the case's clinical improvement due to Metformin use, if there were any **Amagasa et al.** (2011). Lesion size was analyzed using ImageJ Software Version 1.5 3G. A one mm measured by periodontal probe was used as a standardization measuring unit during this study and pixels converted into millimeters and used in statistical analysis. ImageJ has proved cost-effective, computerized, and its results are still reproducible (human errors avoidance). Pictures in ImageJ were standardized by using the same type of camera, lighting, angle and patient distance. To guarantee consistency, this has been confirmed with a periodontal probe **Safadi et al.** (2010).

The present results showed a significant decrease of lesions' surface area after 3 months of Metformin use, whereas placebo group showed an increase in surface area. In accordance, previous preclinical data showed that Metformin significantly reduced the size and number of carcinogens-induced oral tumors and prevented conversion to squamous cell carcinoma from precancerous lesions **Vitale-Cross et al.** (2012). In addition, **Lerner et al.** (2017) reported that multiple dysplastic mucosae continued to present in 3 non-diabetic patients with a history of head and neck cancer. These mucosal

lesions showed complete or partial regression following treatment with Metformin 500 mg twice daily and did not require any additional surgery.

Another clinical indicator was taken in the form of toluidine blue test. **Upadhyay et al.** (2011) reported that toluidine blue application was an affordable and potentially useful tool for the diagnosis of lesions with dysplastic changes.

When we compared the toluidine blue stain intensity at baseline and after 3 months in Metformin vs. placebo group, we found that, there were no statistically significant differences between the two groups, whether at baseline or after 3 months. One explanation could be that most of the study cases were with mild or no dysplasia and the nuclear element attains stain more and appears positive in severe dysplastic lesions, in accordance with the study of **Zhang et al.** (2005). Recent studies by **Awan et al. (2012); Awan et al. (2015); Jajarm et al. (2015) and Rashid & Warnakulasuriya (2015)** suggest that the results of TB are neither accurate nor representative. It was an old theory that was not effective and many authors are in line with the ineffectiveness of TB except for directing the examiner to the best biopsy site as it was observed by ***Gandolfo et al. (2006).*** Chi-square test for Goodness of fit for the distribution of dysplasia grades showed a significant regression of high- grade dysplasia in metformin group compared to placebo and p-value was 0.007. Nevertheless, histological response as an endpoint is infrequently used in oral chemoprevention trials and has yielded inconsistent results **Lodi et al. (2006).**

The secondary outcome was an assessment of the levels of miRNA-31 and miRNA-210 in saliva and tissues in addition to tissue immunohistochemical analysis of cyclin A2 levels. While the occurrence of cancer can be used in chemoprevention research as the primary endpoint **Shin et al.** (2001), the majority of studies have used other indicators as the primary endpoint replacement **Rivarola de Gutierrez et al.** (2014), as adopted in the present study. This is partly because with the prevalence of the cancer as a point of reference, much longer follow up periods, with a huge sample size are mandatory, and still this is not necessarily guaranteed to happen. Actually, malignant transformation has mere possibility, so it may or might not happen. miRNAs are prognostic markers and are graded as an intermediate endpoint signaling tumor cells' proliferation and differentiation, so, we don't have to wait, therefore to see if malignant transformation can occur. **Yang et al.** (2013).

Furthermore, miRNAs were chosen as additional markers for detection of malignant transformation in the present study as they are remarkably stable both in saliva and tissue samples, which offers a great advantage over other biomarker types **Yang et al.** (2013). Also, studies have shown that miRNA profiling can distinguish between normal and cancerous oral tissues, discriminate between different tumor subgroups, and predict therapy outcomes or responses. These results indicate that miRNAs can be used as potential biomarkers for the diagnosis and prognosis determination of OSCC and OPMD **Zhu et al.** (2015); **Harrandah et al.** (2016); **Hung et al.** (2016).

miRNA-31 was chosen in specific because its aberrant expression in saliva was postulated to reflect an early molecular event in the pathogenesis of OSCC **Wu et al.** (2010). Furthermore, several studies had used miRNA- 31 as a prognostic marker in oral leukoplakia such as **Xiao et al.** (2012); **De Sarkar et al.** (2014); **Chattopadhyay et al.** (2016) and **Hung et al.** (2016) . Some of these authors claimed about 80% positivity for miRNA-31 expression was found in OPMD cases with malignant transformation in tissue samples **Xiao et al.** (2012) and **Hung et al.** (2016). **Rothenberg & Ellisen** (2012) concluded that miR-31 could contribute to early oral carcinogenesis via facilitating cell immortalization. A related p53 mutation and VEGF up-regulation, secondary to miR-31 expression, maybe a step further towards more complicated oral keratinocyte neoplastic

induction.
Moreover, a previous study revealed that up-regulated miRNA-31 is critical for epidermal hyperplasia, a stage often followed by dysplasia during malignant progression **Hung et al.** (2014) **.** Alternatively, miR-31 could be related beyond tumor cells to microenvironment modulation, and thus if miRNA-31 expression was not associated with epithelial dysplasia , it remains relevant to cancer so miR-31 may serve as an independent risk factor for OPMD progression regardless of lack of epithelial dysplasia **Yan et al**. (2015).
Thus, when Metformin proves, through the present results, that it suppresses the tissue and salivary levels of miRNA-31 significantly, this might indicate the role it could play in the prevention of carcinogenic changes in OPMDs.
Although both salivary and plasma miRNA-31 were found to be progressively increased following the development of lesions, saliva was chosen to be used in the current study as it has been previously noted that salivary miRNA-31 levels get more elevated relative to plasma levels during the entire process of tumor development. As saliva is the proximal biofluid in direct contact with oral lesions, it is suggested that the release of miR-31 from lesions to saliva would be much more efficient and faster than plasma, which might need to cross layers of connective tissue barriers **Weber et al.** (2010).
Also, miRNA-210 showed significant overexpression in oral cancers as confirmed in the results of **Scapoli et al.** (2010). Again, the present results proved that Metformin can significantly down regulate miRNA-210.
To quantify tissue and salivary miRNA-31 and -210**,** qPCR assay was utilized. The technique of qPCR is one of the most widely used measurement methods for miRNA quantification and expression profiling because of its sensitivity, convenience and short experimental time **Pedersen et al**. (2018)
Moreover, the qPCR system is well known for its precision, speed and broad usage throughout various modern studies, as well as being precise and replicable **Yang et al.** (2013).
On the other hand, Cyclin A, is one among the adverse prognostic factors in oral disorders and oral malignancies **Fraczek et al.** (2008)**.**. It has been known that cell cycle-connected cyclin deregulation and aberrations are involved in tumor growth and development of several cancers **Hahn & Weinberg** (2002) and **Monteiro et al.** (2012). Actually, high expression levels for cyclin A2 have been observed in a high percentage of tumor cells **Hassan et al.** (2002); **Chen et al.** (2003); **Saarilahti et al**. (2003) and **Fraczek et al**. (2008).
Cyclin A2 expression was evaluated using immunohistochemistry (IHC), which is considered to be an advanced form of histopathology which helps to form a diagnosis whenever ordinary examination is not well trusted. IHC is often used in situations where a presence or absence of certain proteins can form a basis for a diagnosis. It can also be used to distinguish between two different disease processes that may otherwise appear similar to the pathologist. The advantages of IHC include: It is possible to use fresh or frozen tissue samples for IHC, it is well-established and readily available, the cost is relatively low, it has a fast turn-around time. Moreover, because no live infectious agents are involved, the risk to human health is minimal.
In the present work, cyclin A2 level proved to decline significantly after 3 months of Metformin administration. The comparison plotted between groups regards cyclin A2 showed that the decrease in cyclin A2 levels was significantly different between groups, in favor of Metformin.
In brief, the present results point out to the fact that Metformin is able to manipulate more than one molecular target molecule, all of which have some level of participation

in the pathways of carcinogenesis. Through interacting with and down regulating the expression of key molecules interplaying within the regulatory machineries of the cell cycle and epigenetic control of cell proliferation, Metformin seems to exert its anticancer effect via decreasing the proliferation rate of OPMD lesion cells. Such results add molecular evidence to that presented by our concomitant trial **Abdel-Azim et al. (2021).** The results of the present clinical trial thus foster the hope to achieve oral cancer chemoprevention in high-risk patients through the use of Metformin.

CONCLUSIONS

1- Metformin HCL administration at a dose of 500 mg/ day might be of real applicability as a cancer chemopreventive agent in OPMDs.
2- Metformin HCL administration has the potential to reverse one of the most documented epigenetic oncogenic pathways, namely the one related to miRNA-31 and miRNA-210 upregulation.
3- One of the possible in vivo mechanisms of cancer chemoprevention afforded by metformin hydrochloride is the inhibition of nuclear cyclin A2 expression and downregulation of miRNA-31 and -210.

RECOMMENDATIONS

1- More clinical trials with Metformin hydrochloride, with different periods of administration and longer patient follow up are recommended to be able to point out the most favorable and applicable regimen for the utmost level of oral cancer chemoprevention.
2- Deeper digging into all possible mechanisms of cancer chemopreventive mechanisms related to Metformin hydrochloride is needed to unravel the whole picture of the drug's capabilities and most beneficial applications.
3- Clinical trials with other candidate drugs with epigenetic effects are recommended in the field of oral cancer chemoprevention.

Author contributions

Professor Fat'heya Mohamed Zahran: design of study and final revision, Professor Gihane Gharib Madkour: biostatistics oversight, manuscript preparation, and editing, Professor Heba Ahmed Farag: pathology, IHC, manuscript preparation, and editing, Professor Olfat Gamil Shaker: miRNA analysis.

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