RESEARCH ARTICLE

Analysis of Metabolic Regulators *PGC1-α* and *PGC1-β* in Oral Squamous Cell Carcinoma with and without Hyperglycemia

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Abstract

   Aim: To assess the expression of *PGC1-α* and *PGC1-β* in Oral squamous cell carcinoma in the presence and absence of hyperglycemia. **Materials and Methods:** Fresh tissue samples were collected from 14 well differentiated OSCC patients with hyperglycemia, 14 OSCC patients without hyperglycemia and 14 healthy controls and subjected to quantitative real-time PCR to assess expression of *PGC1-α* and *PGC1-β*. The relative gene expression of *PGC1-α* and *PGC1-β* was calculated using the double delta Ct method. A two-fold difference was defined as over or under- expression. To further evaluate clinicopathological association, Independent t-test was employed. **Results:** The expression of both *PGC1-α* and *PGC1-β* were increased in OSCC patients when compared to healthy controls and similar findings were observed on calculating the fold change healthy controls and OSCC study groups. On assessing the expression of target genes within study groups, they did not present with significant fold change and the hyperglycemic status of the individual did not contribute to the expression of the target genes as P value obtained for *PGC1-α* and *PGC1-β* were >0.05. **Conclusion:** The hyperglycemic status of the individual does not influence the expression of *PGC1-α* and *PGC1-β* in OSCC tissues and the cause for over-expression of the study targets in OSCC tissues must be further evaluated to assess their potential as possible candidates for targeted therapy in OSCC patients.

Keywords: Diabetes mellitus- Hyperglycemia- Oral cancer- *PGC1-α- PGC1-β*

Introduction

   Oral squamous cell carcinoma (OSCC) is the most common malignancy globally and it has a diverse distribution around the world. Its prevalence in unindustrialised countries is much higher when compared to the Western world. In India, it is the most common cancer affecting males (Bugshan and Farooq, 2020). Various etiologic factors such as tobacco, alcohol and UV exposure have been identified for OSCC initiation. The modern advancements in the field of DNA analysis have led to the identification of numerous genetic mutations that can initiate carcinogenesis in the oropharynx and oral mucosa; nonetheless, a specific gene has not been identified for the development of OSCC (Muttagi et al., 2012).

   Another disorder that shares its potential etiologic factors , tobacco and alcohol consumption, with OSCC is diabetes mellitus. Diabetes mellitus (DM) is a disease that mainly affects the metabolism of the individual and presents as increased blood sugar levels due to lack of response or insufficient production of a hormone, namely,

insulin, whose primary function is to stimulate glucose uptake by various cells to regulate the blood glucose level and aid in the inhibition of hepatic glucose production. The most common subtype of DM is Type II DM wherein the pathology is primarily due to the lack of response by the body to the production of insulin. A major consequence of DM is hyperglycemia (HG) that adversely affects the normal metabolic homeostasis of an individual (Khan et al., 2019). Chronic hyperglycemia can aid in development of various systemic disorders; wherein oral cavity can also be affected. . Many studies conducted in recent years have suggested the possible influence of Diabetes mellitus in the initiation and progression of potentially malignant oral disorders that if left untreated may progress to frank OSCC (Cicmil et al., 2017). But the influence of diabetes mellitus in OSCC initiation and progression is not explored.

   The hallmarks of cancer that are explored extensively in recent times include dysregulation of cellular energy metabolism, genomic instability and mutation and chronic inflammation. that aids in tumor progression. Peroxisome proliferator-activated receptor gamma coactivator (PGC1) is a central metabolic modulator involved in all these three

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hallmarks of cancer progression (Bost and Kaminski, 2019). It has also been identified as a catalyst in many metabolic processes that are affected in Diabetes mellitus (Wu et al., 2016).

The members of the PGC1 family includes PGC1-α, PGC1-β and PGC- related coactivator (PRC). The two co-activators that are implicated in both the pathogenesis of DM as well as in metabolic pathways of cancers mentioned above are PGC1-α and PGC1-β (Ramachandran et al., 2012).

Thus, the current study aims to assess the alteration in the expression of *PGC1-α* and *PGC1-β* in OSCC when compared to normal healthy controls and also if the hyperglycemic condition of the individuals impacts the expression of PGC1-α and PGC1-β.

Materials and Methods

Sample Size Calculation

Using the sample size calculator (G* Power Version 3.1.9.2) a minimum sample size of 5 in each group was calculated.

Patient selection

The study was approved by the Institutional Review Board of SRM Institute of Science and Technology, Ramapuram, Chennai (IRB Approval no: SRMDC/IRB/2017/MDS/No.602). A total of 42 patients from SRM Dental College, Ramapuram between 2019-2021 were enrolled in the study. Written informed consent was obtained from all participating patients. They were categorized into three major groups: normal controls (n=14), OSCC patients without hyperglycemia (n=14) and OSCC patients with hyperglycemia (n=14). The inclusion and exclusion criteria of the groups were as follows:

GROUP I: Healthy controls
Inclusion criteria

Healthy adults with HbA1c levels between 4% to 5.6% and free of any potentially malignant oral disorder and Oral squamous cell carcinoma.

Subjects free of systemic diseases.
Exclusion criteria

Subjects with HbA1c levels 5.7% and higher and with systemic diseases.

Subjects diagnosed and/or treated for potentially malignant oral disorders, Oral squamous cell carcinoma.

GROUP II: Subjects with Oral squamous cell carcinoma without Type II Diabetes mellitus
Inclusion criteria

Subjects recently diagnosed of Oral cancer and had not undergone any treatment

Patient free of systemic diseases and with HbA1c levels between 4% and 5.6%.

Exclusion criteria

Subjects diagnosed and/or treated for Oral squamous cell carcinoma.

Subjects diagnosed with systemic diseases
Subjects with HbA1c levels 5.7% and higher.

GROUP III
Subjects with Oral squamous cell carcinoma with Type II Diabetes mellitus
Inclusion criteria

Subjects recently diagnosed of Oral cancer and had not undergone any treatment.

Subjects with HbA1c levels between 4% and 5.6%
Exclusion criteria

Subjects diagnosed and/or treated for Oral squamous cell carcinoma.

Subjects diagnosed with systemic diseases
Subjects with HbA1c levels 5.7% and higher.

qPCR analysis

All the samples were collected and immediately stored in -80°C. The samples were then processed and subjected to qPCR reaction as follows.

RNA was extracted from each tissue sample using RNA isoPlus reagent (Takara Bio Inc, Shiga, Japan) according to manufacturer protocols and its concentration was assessed using a Nanodrop™ (Thermo Scientific, Massachusetts, USA) . Briefly cDNA was synthesized from 200 µl of total RNA.

The primers used in real-time PCR reaction (Sigma-Aldrich, St. Louis, MO, USA) were formulated based on the sequences identified by Silvennoinen et al., (2015), prepared in Bio Corporals, Chennai and are listed in Table 1 . All PCR reactions were performed using Light cycler 96 (Roche, Basel, Switzerland). For each PCR run, a master mix was prepared on ice with 10 µl TB Green® Premix Ex Taq™ II (Takara Bio Inc, Shiga, Japan), 0.8 µl forward and reverse primers, 2 µl cDNA and 6.4 µl nuclease free water for a total of 20 µl. The *housekeeping* gene used in the reactions was GAPDH.

All samples were run in triplicates. The thermal cycling conditions were at initial 95°C for 30 seconds, 40 cycles at 95°C for 35 seconds and 60°C for 1 minute.

Relative gene expression analysis

The relative expression of the genes was obtained by comparing the PCR product from each OSCC sample to healthy controls and comparison between PCR product of OSCC samples with and without hyperglycemia was also carried out. The method used for calculating the relative gene expression of *PGC1-α* and *PGC1-β* was done using the "double delta Ct" method (Livak and Schmittgen, 2001). A gene expression in disease tissue less than 2-fold of the normal counterpart was defined as underexpression and one with greater than 2-fold the normal counterpart was defined as overexpression.

Statistical analysis

Independent t- test was done to compare the difference in gene expression between different groups. All p-values were 2-sided; $p \leq 0.05$ was considered statistically significant.

Results

Descriptive data of study groups

The characteristics of the 42 patients enrolled in the study are described in Table 2. All study groups were predominantly composed of male patients. In total, 27 male patients and 15 female patients were included in the study. None of the participants in the healthy control groups reported with a habit history. But all participants in the OSCC study groups reported at least one habit history (smoking, smokeless tobacco, alcohol) for a minimum of 2 years.

Quantification of PGC1-α in study groups

When comparing the level of PGC1-α between healthy group tissue and OSCC tissues (Figure 1), it was observed that the expression level increased significantly in both OSCC with diabetes and OSCC without diabetes in comparison to healthy controls.

Quantification of PGC1-β in study groups

When comparing the level of PGC1-β between healthy group tissue and OSCC tissues (Figure 2), it was observed that the expression level increased significantly in both OSCC with diabetes and OSCC without diabetes in comparison to healthy controls.

Relative gene expression analysis

The assessment of gene expression between OSCC tissue and healthy controls is depicted in Table 3.

On assessment of OSCC tissue without hyperglycemia, there was significant increase in expression of both the genes *PGC1-α* and *PGC1-β* when compared to healthy counterparts, namely 38.5 times and 38.07 times respectively.

Similar results were obtained in OSCC tissues with hyperglycemia when compared to their normal counterparts as PGC1-α levels were 35.8 times more than normal and PGC1-β levels were 29.38 times more than normal. Therefore, the expression of both *PGC1-α* and *PGC1-β* was significantly higher than the normal tissue, and the upregulation was slightly higher

Table 1. Names and Sequences of Oligonucleotides Used for Real-Time RT-PCR Analysis

Primer name	Sequence
PGC1-α[1] forward	5'-AGCCTCTTTGCCCAGATCTT-3'
PGC1-α reverse	5'-GGCAATCCGTCTTCATCCAC-3'
PGC1-β[2] forward	5'-GAGTCAAAGTCGCTGGCATC-3'
PGC1-β reverse	5'-AACTATCTCGCTGACACGCA-3'

[1] PGC1-α, Peroxisome proliferator-activated receptor gamma coactivator (PGC1) alpha; [2] PGC1-β, Peroxisome proliferator-activated receptor gamma coactivator (PGC1) beta

Table 2. Demographic Details of Study Participants

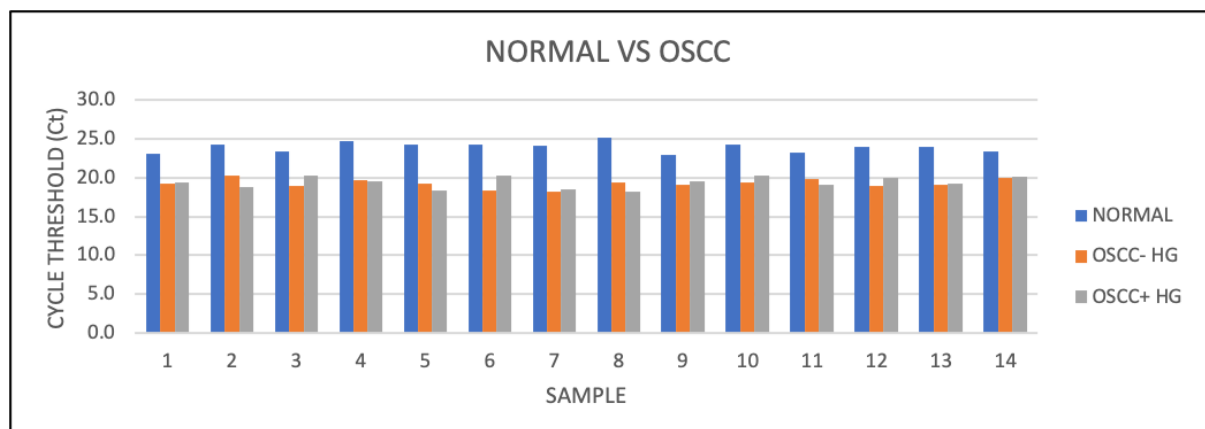| Characteristic	Healthy Controls N (%)	OSCC [1]without hyperglycemia N (%)	OSCC with hyperglycemia N (%)
Gender			
Male	8 (57.1)	9 (64.3)	10 (71.4)
Female	6 (42.9)	5 (35.7)	4 (28.6)
Age			
20-40 years	10 (71.4)	2 (14.3)	2 (14.3)
41-60 years	4 (28.6)	10 (71.4)	10 (71.4)
61-80 years	0	2 (14.3)	2 (14.3)
Habit			
Smoking	0	9 (64.3)	10 (71.4)
Smokeless tobacco	0	14 (100)	14 (100)
Alcohol	0	9 (64.3)	8 (57.2)

[1] OSCC, Oral Squamous cell carcinoma

Figure 1. Comparison of Expression of PGC1-α in Healthy Tissue, OSCC Tissue with and without Hyperglycemia
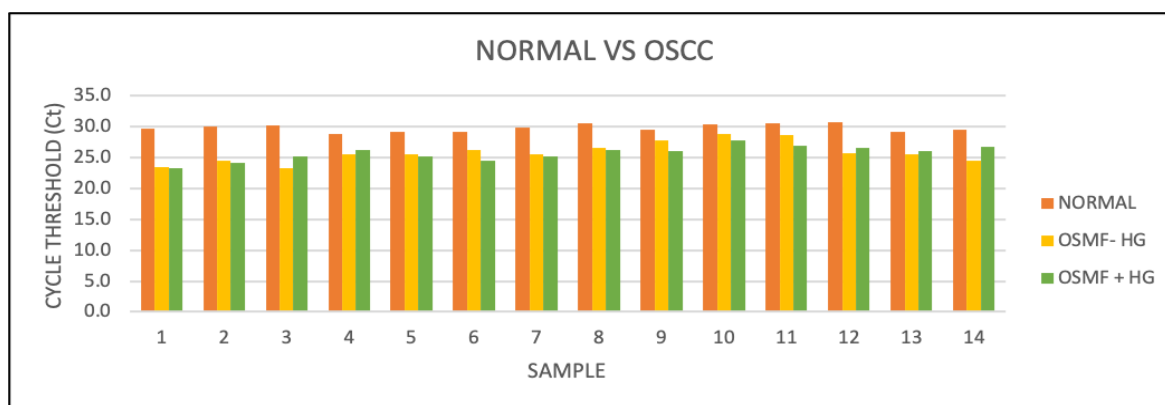
Figure 2. Comparison of Expression of PGC1-β in Healthy Tissue, OSCC Tissue with and without Hyperglycemia

Table 3. Average Fold Change of Gene Expression between Study Groups

Group A	Group B	Average fold change in PGC1-α[1]	Average fold change in PGC1- β[2]
Normal	OSCC[3] without hyperglycemia	37.5	38.07
Normal	OSCC with hyperglycemia	35.8	29.38
OSCC without hyperglycemia	OSCC with hyperglycemia	1.11	1.41

[1] PGC1-α, Peroxisome proliferator-activated receptor gamma coactivator (PGC1) alpha; [2] PGC1- β, Peroxisome proliferator-activated receptor gamma coactivator (PGC1) beta; [3] OSCC, Oral Squamous Cell carcinoma

in non hyperglycemic patients when compared to their hyperglycemic counterparts.

On comparing relative gene expression between OSCC study groups with and without hyperglycemia, there was upregulation of both PGC1-α and PGC1-β in OSCC tissues without hyperglycemia. The level of PGC1-α increased by 1.11 folds while the level of PGC1-β increased by 1.41 folds in OSCC tissue without hyperglycemia. As the alteration in gene expression was less than 2 fold, the change in the relative gene expression was not significant.

Statistical analysis

Statistical analysis of the data was performed using SPPS Software, Version 23.0. The descriptive statistics such as mean and standard deviation [SD] were calculated for individual groups. The confidence interval was set at 95%.The comparison of gene expression between healthy controls and OSCC study groups assessed using

Independent t-test and summarised in Table 4.

On comparing the data obtained for PGC1-α levels between Healthy controls and OSCC subjects with and without diabetes using Independent t-test, the P value was <0.05 (P=0.000) in both the comparisons, therefore the results obtained were found to be statistically significant.

Similarly, on comparing the data obtained for PGC1- β levels between Healthy controls and OSCC subjects with and without diabetes using Independent t-test , the P value was <0.05 (P=0.000) in both the comparisons , therefore the results obtained were statistically significant

Thus, the above mentioned statistical data analysis between healthy controls and the study group suggests that the hyperglycemic index of the patient along with the disease has a contribution in the expression of PGC1-α and PGC1- β in all study groups when compared to healthy controls.The data comparison within OSCC study groups for PGC1-α and PGC1- β are tabulated in Table 5.

On comparing the data obtained for PGC1-α levels

Table 4. Statistical Analysis between Healthy Controls and OSCC Study Groups

Gene	Group 1	Group 2	Mean difference	Std. error difference	P value
PGC1-α[1]	Healthy controls	OSCC[2] without diabetes	4.393	0.319	0.000*
	Healthy controls	OSCC with diabetes	4.265	0.338	0.000*
PGC1- β[3]	Healthy controls	OSCC without diabetes	3.612	0.576	0.000*
	Healthy controls	OSCC with diabetes	3.783	0.478	0.000*

* Found to be statistically significant; [1] PGC1-α, Peroxisome proliferator-activated receptor gamma coactivator (PGC1) alpha; [2] OSCC, Oral squamous cell carcinoma; [3] PGC1- β, Peroxisome proliferator-activated receptor gamma coactivator (PGC1) beta

Table 5. Statistical Analysis within OSCC Study Groups

Gene	Group 1	Group 2	Mean difference	Std. error difference	P value
PGC1-α[1]	OSCC[2] without diabetes	OSCC with diabetes	-0.128	0.249	0.612
PGC1- β[3]	OSCC without diabetes	OSCC with diabetes	0.171	0.556	0.761

[1] PGC1-α, Peroxisome proliferator-activated receptor gamma coactivator (PGC1) alpha; [2] OSCC, Oral squamous cell carcinoma; [3] PGC1- β, Peroxisome proliferator-activated receptor gamma coactivator (PGC1) beta

between OSCC subjects with and without diabetes using Independent t-test, the P value was >0.05 (p=0.612) , therefore the results obtained were not statistically significant. Similar statistically insignificant results were obtained on comparison of data obtained for PGC1- β levels between OSCC subjects with and without diabetes using Independent t-test as the P value obtained was >0.05 (p=0.761).

Thus it can be understood from the above findings that, the hyperglycemic status influences gene expression of the target genes when progressing from an apparently healthy tissue to OSCC. But, the influence of the hyperglycemic status in altering the gene expression of *PGC1-α* and *PGC1-β* in an established OSCC lesion is not significant.

Discussion

Cancer plays a significant role in its contribution for morbidity and mortality worldwide. Among the different types of cancers, oral cancer is found to be most prevalent in the Eastern world, possibly due to increased prevalence of habits such as smoking and tobacco chewing that initiate cancer. Along with cancer, another autoimmune disorder that affects the quality of life of an individual is Diabetes mellitus. A state- wise evaluation of diabetes prevalence in India suggested that prevalence increased from a moderate 5.5% in 1990 to a giant leap of 7.7% in 2016. Also, Tamil Nadu has the highest prevalence of Diabetes mellitus when compared to other states of India (Tandon et al., 2018).

Both these conditions have been identified to have genetic predisposition as a major risk factor. One of the first studies to identify a possible genetic predisposition for Type II Diabetes mellitus was done in 2000, that found a specific polymorphism in PPARgamma which had a significant influence on Type II diabetes occurrence (Altshuler et al., 2000). PPARgamma is a member of a large group of nuclear receptors that require transcriptional co-activation by molecules such as PGC1-α and PGC1-β to perform their function in fatty acid oxidation and regulation of energy metabolism. Multiple studies in recent times have found a possible role of these co-activator molecules in energy metabolism and thus, a possible impact in cancer initiation and progression (Liang and Ward, 2006).

A meta- analysis conducted suggested that participants with Type II Diabetes mellitus were at an increased risk of developing premalignant lesions and cancerous lesions when compared to their non-hyperglycemic, healthy controls (Gong et al., 2015).

The present study, first of its kind, aims at exploring whether in a disease condition such as OSCC, the expression of metabolic co-activator molecules PGC1-α and PGC1-β is altered and also, if the hyperglycemic status of the patient contributes for the same.

On assessing the results of the present study we observe that, the expression of PGC1-α and PGC1-β was upregulated in both OSCC study groups when compared to healthy controls (p=0.000). The relative gene expression analysis also presented with a similar result wherein OSCC patients without diabetes had a fold change of 37.5 in

case of PGC1-α and 38.07 in PGC1-β when compared to healthy controls. Also, relative gene expression in OSCC patients with diabetes presented a fold change of 35.8 and 29.38 in PGC1-α and PGC1-β respectively when compared to healthy controls. This increase in PGC1-α and PGC1-β expression in OSCC patients can be explained by finings of study conducted in 2010 by Theocharis S et al in mobile tongue SCC patients wherein PPAR gamma levels were significantly increased in cancer tissues when compared to healthy controls (Theocharis et al., 2011). The slight reduction in *PGC1-α* expression level in the presence of diabetes mellitus can be explained by the findings of the study conducted by Mootha. V et al, where levels of *PGC1-α* were found to be reduced in patients with Diabetes mellitus when cell lines were cultured from muscle biopsy of recently diagnosed Diabetes mellitus individuals in mouse models (Mootha et al.,2003). Also, the slight downregulation of PGC1-β in diabetic patients can be explained by the results of study performed by Patti.ME et al, that exhibited that there was significant decrease in PGC1-β mRNA levels of skeletal muscle biopsies taken from diabetic and prediabetic individuals (Patti et al., 2003).

When assessing the gene expression within the OSCC study groups with and without diabetes it was observed that the fold change though present was not significant as they were less than 2 folds. Added to this, statistical analysis also presented with similar results wherein the increase in expression of target genes was not statistically significant as P>0.05. These results suggest that the hyperglycemic status of the individual does not influence the expression of *PGC1-α* and *PGC1-β*. Since the current study has a unique study design, studies with similar parameters were not obtained in the literature search. Even though there is a decrease in the PGC1 α and PGC1-β in OSCC patients with diabetes in the present comparison, still the level of PGC1 α and PGC1-β in OSCC patients with diabetes is statistically upregulated when compared to healthy controls. This significant increase in the level of PGC1 α in cancer tissue might be explained by a study conducted in cancer tissue of a different organ , namely, prostate cancer. The study conducted by Massie et al., (2011) in prostate cancer cell lines using genomic studies and metabolomic profiling suggested that increased PGC1 α activation and subsequent stimulation of androgen receptor was pivotal for maintaining central metabolism and cell survival in prostate cancer. Similarly, the increase in PGC1-β can be explained by the mice model studies conducted by Roy et al., (2020) that suggest that hypoxia induces alteration in mitochondrial biogenesis processes and this change is initiated in cancer cells by a pro- oncogenic transcription factor, MYC, which increases the expression of PGC1-β that in turn activates PGC1 α to act on reactive oxygen species to aid in survival of the cancer cells.

Thus, the study results suggest that the expression of the target genes were increased in disease subjects without diabetes mellitus when compared to other groups. Even though, the disease group with diabetes mellitus had a reduced expression when compared to disease groups without diabetes, their levels were increased when compared to healthy controls. However, it is yet to be

explored whether this alteration in expression is a cause or effect of the disease condition observed in the study groups. Thus, further research is needed to explore the role of PGC1-α and PGC1-β in diabetic and non-diabetic OSCC individuals to assess their potential as candidates for targeted therapy in oral cancer.

It must be noted that the current study has its own set of limitations. The single centre approach and the small sample size due to the pandemic situation reduces the impact of the study results. Also, all the OSCC samples in the current study were well-differentiated OSCC and other grades of OSCC were not evaluated in the study. Age and sex matching could not be maintained among all the groups. Therefore future studies must be done with a multicentric approach, larger sample size with age and sex matching across all groups and incorporating more parameters such as diabetes history of the participants, drug history, site of oral lesion and treatment history of diabetic participants to validate the findings of the current study and assess their potential for targeted therapy.

In conclusion, the current study outcomes reveal that the expression of both *PGC1-α* and *PGC1-β* is upregulated in OSCC when compared to healthy controls. However, this upregulation of PGC1-α and PGC1-β is slightly reduced in OSCC in the presence of diabetes when compared to OSCC in the absence of diabetes. Hence, it is yet to be explored whether this alteration in expression is a cause or effect of the disease condition observed in the study groups. Therefore, further studies are required to understand their mechanism in OSCC tissues considering the hyperglycemic status of the patient.

Author Contribution Statement

TTP, AR, RR, KR conceived and designed the study; TTP searched the databases, screened titles, abstracts and full text studies included; AR and TD performed data analysis and data interpretations; TTP, AR, TD and GN drafted the initial manuscript. All authors performed critical revision of the manuscript and approved the final version of the manuscript.

Acknowledgments

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