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Detection of TTV and EBV in Type II Diabetic Patients with Chronic Periodontitis Patients - A Pilot Study

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Abstract: ***Background: Periodontitis and diabetes share common relationship in that both are chronic inflammatory conditions. Periodontitis is multifactorial in which the bacterial-host response is the most accepted etiology. With the discovery of association of various viruses such as EBV, CMV and TTV viral implications have been hypothesized. Periodontitis is an established risk factor for diabetes and progression of periodontitis more in diabetic patients than in healthy individuals. The exact mechanism of periodontitis is still being researched. This study examined the occurrence of EBV, TTV and their coexistence in chronic periodontitis patients with and without Type II diabetes. Methods: 30 patients were selected and divided into 2 groups of 15 each consisting of control (chronic periodontitis) and test (chronic periodontitis with type II diabetes). Conventional PCR was used to detect the presence of EBV, TTV from the gingival biopsies. The clinical parameters were measured using modified CPI index and the results were subjected to various statistical analysis. Results: EBV was detected in 13.3% of chronic periodontitis patients and 20% of diabetic patients. (p = 0.62) TTV was seen only in diabetic patients and was highly significant with p = 0.00. Coexistence of EBV and TTV was found in 20% of diabetic patients (p = 0.33). Conclusion: TTV was found in the gingival biopsies and was significantly associated in chronic periodontitis patients with type II diabetes. No significant coexistence of TTV and EBV could be established.***

Keywords: TTV, EBV, Type II diabetes, PCR

1. Introduction

Periodontitis is a chronic multifactorial disease that progresses by destruction of supporting structures of teeth like cementum, alveolar bone and periodontal ligament. (1) The pathogenesis of periodontitis is considered to have complex interactions between microbial factors, host factors, and various environmental factors. Bacterial-host response has been implicated to be the main etiological factor for the disease pathogenesis. The progressive course of periodontitis typically cannot be explained based only on this mechanism, therefore various hypothesis are still being formulated to explain its etiology, of which viral-bacterial hypothesis is one of them.(1) Studies on a viral cause for periodontitis have marked a turning point in periodontal research, which until recently was centered almost exclusively on a bacterial etiology. Still, the progress has been very slow in this area even with the advanced technologies as viruses are a challenge in detection and treatment when compared to bacteria.

It has been found that human adults are carriers of human cytomegalovirus and Epstein–Barr virus and both of them have been frequently seen with periodontal diseases.(2) Recently, discovery of Torque Teno Virus (TTV) in periodontitis has rekindled the interest in the role played by this virus in periodontitis.(3) It belongs to Circoviridae family and genus Anellovirus and was found in gingival biopsies, subgingival plaque of periodontitis patients. Also, TTV has been found to vary in various demographic profiles. (4) Epstein Barr virus was found to enhance the replication of Torque Teno virus in a study which was said to be one of the mechanisms behind the pathogenesis. (4,5) Coexistence of TTV with EBV and its relationship with chronic periodontitis and aggressive periodontitis has been studied but is still controversial. Though, Epstein Barr virus have been found in diabetic patients but so far, there have been no studies done on Torque Teno Virus and their coexistence in patients with Diabetes.

2. Material and methods

This was an explorative study to check the relationship between Torque Teno virus, Epstein Barr virus in gingival tissue biopsies of 30 patients comprising of 15 patients in control group (chronic periodontitis without Type II diabetes) as well as test group (chronic periodontitis with Type II diabetes) in the age group of 30-60yrs. The patients for this study were selected from the outpatient section, Department of Periodontology, JSS Dental College and Hospital, Constituent College of JSS AHER, Mysore. The research protocol was approved by the Institutional Review Board at JSS Dental College, Mysore [IEC no: 08/2018] prior to commencement of the study. An informed written consent was obtained from all the subjects and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Patients were included within the age group of 30-60 years both males and females diagnosed with severe chronic periodontitis (CAL ≥ 5mm) (6) for both the groups, Type II Diabetes patients with HbA1c < 9 for test group, periodontally involved tooth that was indicated for extraction and patients who werewilling andgave informed

consent. Patients were excluded who had undergone periodontal treatment in last 6 months, with diabetes associated complications, smokers, who were on immunosuppressive drugs, pregnant and lactating mothers.

Collection of data:

The clinical parameters such as bleeding on probing, clinical attachment level, probing pocket depth were recorded to assess periodontal condition in the included subjects using modified CPI index [7]. A proforma was then given to record the details of the patients including the duration of Diabetes and the levels of glycated haemoglobin for diabetic patients. This was performed before gingival biopsy was taken.

A gingival tissue biopsy of size approximately 4*2*2 mm was taken from the teeth that were indicated for extraction due to periodontal reasons. The specimen was then transported in a sterile container containing 10% formalin and was then made into paraffin blocks.

PCR analysis:

DNA was extracted by using a reagent kit according to manufacturer's instructions. Specific primer sequences were used for identification of EBV, TTV. Polymerase chain reaction (PCR) amplification was done using a thermal cycler (Applied Biosystems, USA). Primer sequence used for EBV was: 5'-AGC ACT GGC CAG CTC ATA TC -3' as forward,
5'-TTG ACG TCA TGC CAA GGC AA -3' as reverse. TTV primer used was: 5'-GCTACGTCACTAACCACGTG -3' as forward, 5'-CTBCGGTGTGTAAACTCACC-3' as reverse.

Reaction mixture was prepared using AMPLIQON RED 2X Mastermix.10µl Ampliqon Master mix, 0.5 µl (5 p mole) of EBV/TTV (Forward primer), EBV/TTV (Reverse primer), 2 µl Template DNA and water was added to make final volume to 20 µl and PCR was performed using thermal cycler (Applied Biosystems, USA). The amplified products were visualized on a 2% agarose gel intercalating ethidium bromide dye and were subjected to electrophoresis at a current of 16A for 2 hours. The bands were recorded using Gel documentation system (Major Science, USA). [8,9]

Specific amplified product was identified by comparing the band size of test samples with 100 base pair DNA ladder (marker). Amplified product of 326 base pair was identified which corresponds to EBV and amplified product of 199 base pair was identified which corresponds to TTV. [10]

3. Statistical analysis

The statistical package for social science (version 22) was used to perform statistical analysis. Chi square test was used to check the association between TTV and EBV in test group and TTV, EBV with various clinical parameters. Descriptive statistics was used to check for mean age group and gender variation, prevalence of the virus in both the groups. Statistical significance was fixed at $p \leq 0.05$.

4. Results

A total of 30 patients with a mean age of 49 ± 7.6 in the control group (n=15) and 49.5 ± 6.8 in the test group (n=15) was considered in the study and descriptive statistics was used to compare the mean age group and gender in both groups. It was found that age was not significant (p = 0.84), nor was gender found to be statistically significant (p= 0.71) in both the groups. [Table 1]

Table 1: Descriptive statistics

Groups	Mean age ± Standard deviation	Gender		Prevalence of TTV	Prevalence of EBV
		Females	Males		
Control (n=15)	49 ± 7.6	7	8	0%	13.3%
test (n=15)	49.5 ± 6.8	8	7	80%	20%
P value	0.84	0.71		0.00	0.62

Presence of TTV in gingival tissue biopsy was detected in 12 patients out of 15 (80.0%) in the test group and was highly significant with p = 0.00 in the test group only. TTV was not detected in the control group. Whereas, EBV was detected in 2 patients (13.3%) in the control group and 3 patients (20%) in the test group which was not significant. (p value= 0.62)

TTV was not detected in the control group, EBV was found in 2 patients out of 15. Whereas, in the test group TTV and EBV coexisted in 3 patients compared to no patients when TTV was not detected. This association was found to be not significant in test group. (p = 0.33)

5. Discussion

Periodontitis is a multifactorial, chronic disease that progresses by the destruction of supporting structures of teeth such as cementum, alveolar bone, and periodontal ligament. The main cause of periodontitis is the oral biofilm, with multiple microorganisms present such as bacteria and viruses. Various viruses have been found to play a role in periodontitis by infecting the inflammatory cells of the periodontium. They also interfere with the immune responses through immune modulators and are therefore seen more frequently in periodontally diseased sites compared to the healthy sites. [11]

Periodontitis has been established as a risk factor for diabetes and is more commonly seen in the diabetic patients compared to healthy individuals. [12] Hence there was a necessity to study the viral etiology involved in the progression of periodontitis in the diabetics, so this research was undertaken.

Various studies by Contreras A et al, Rotola et al [13,14] have emphasized on the role of EBV and other viruses in the initiation and progression of periodontal disease. In a meta-analysis by Zilong Gao et al [15], he suggested that the presence of EBV in periodontitis is still controversial. The mechanism by which EBV acts in periodontitis patients is that it promotes subgingival attachment and colonization of periodontopathic bacteria and the compounds released from them is responsible for the activation of latent EBV thereby causing destruction. It also infects and alters the functions of monocytes, macrophages, and lymphocytes in periodontitis lesions. [16] Even though, EBV has been detected more in type I diabetes patients there is less literature available on the presence of EBV in Type II diabetes mellitus. In our

study also, even though EBV was detected among 3 out of 15 patients, it was not significant and in the healthy individuals it was seen in 2 out of 15 which was also not significant.

Slots [17] in his review article has reported a prevalence of EBV ranging from 3% to 81% in chronic periodontitis patients in systemically healthy subjects. In the present study it was found that EBV was detected in only 13.3% in healthy individuals with CP. Similar results were obtained by various other studies by Nibali L et al, Ding F et al. [18,19] Whereas, 20% of the patients in the diabetic group were detected with EBV, which was similar to the results obtained by a study done by Dworzanski et al [20] who found a prevalence of 25.4%. This wide range could be explained by the differences in the study groups which were considered in the study, the demographic characteristics of the study subjects and the different detection methods used. In our study conventional PCR was used that only detects the presence and absence of the virus as opposed to RT-PCR used in other studies which is more sensitive and provides a quantitative data. The samples which are sensitive to detection of viruses are subgingival plaque and biopsy compared to GCF and saliva. Hence, in the current study gingival tissue biopsy was used for detection of EBV and TTV. Some difficulties in virus extraction have been found from tissue samples as well, such as: i) difficulty to extract specimen ii) small and variable size of tissue sample iii) probability of contamination of samples iv) gingival biopsy was taken from the extraction site which might not be the representative of other areas which might have harboured the virus. All these reasons could explain the lower detection rates of the virus. [21]

In the current study only controlled diabetic patients were considered with HbA1c $\leq$ 9 to exclude any possibility of patients developing diabetic complications. This might have led to less detection of EBV in the test group. EBV was found to be more prevalent in diabetic patients with poor glycaemic control (HbA1c $\geq$ 10) in a study by Casarin et al which is contradictory to our findings. [22]

Imbronito et al [23] found that EBV was present in deeper pockets in nearly 46.7% of the subjects with chronic periodontitis. In this study, contradictory results were found which suggested that EBV was more prevalent (80%) in the shallow pockets (4-5mm) compared to deeper pockets (20%) in both the test group as well as control group but it was not significant (p = 0.24).

Zhang Y et al in 2016[24] carried out the first study that detected novel human Anellovirus species of TTV in the gingival tissue from chronic periodontitis patients. Various studies have been carried out that detected the presence of TTV in various systemic diseases such as in Hodgkin's Lymphoma, nephropathy and immunocompromised patients. [25,26,27] Very few researches are present that have evaluated TTV and EBV titres in diabetics with periodontitis. Also, According to Borkosky et al [28] EBV was found to stimulate the replication of TTV. Hence, this study was carried out to check the EBV, TTV titres as well as the coexistence of them.

In our study, EBV was not compared with CMV but it was found to have coexisted with TTV in 3 out of 15 patients in the diabetic group which was not significant (p = 0.33). Study was carried out by Tian Yu et al [29] which found that the coexistence rates of EBV and TTV were significantly higher in AP and CP groups. (p=0.01) In the control group TTV was not detected hance the coexistence could not be studied.

Various studies have confirmed the role of TTV in causing periodontitis. [30,31] Contradictory to this, in the present study, TTV was not found in the chronic periodontitis patients but was seen in 12 out of 15 patients (80%) in the diabetic group which was highly significant (p=0.00). The possible explanation being TTV has shown to alter the expression of IL-6 through Toll-like receptor 9 and also mediate the response to IFN and other cytokines. These changes in the host immunoreactivity might influence the evolution of the diabetes and its chronic complications. [25]

Limitations of the study: In the current study, virus was detected using conventional PCR that only detected the presence and absence of the virus, also there was a lack of systemically healthy group without chronic periodontitis. And only controlled Type II diabetes mellitus patients were considered.

6. Conclusion

Higher prevalence of TTV in controlled Type II diabetic patients was seen which was significant compared to control group. This suggests that TTV replicates actively in the immunocompromised patients. The current data indicates that the TTV acts more likely as an aggravating factor in the evolution of disease in people with various pathologies and does not cause the disease itself. Further studies using larger population, advanced viral detection methods should be carried out in well controlled, poorly controlled diabetes patients to confirm its association with Type II diabetes mellitus patients. Within the limits of the study, it can be concluded that TTV can act as a biomarker for progression of disease in patients with chronic periodontitis with Type II diabetes, but further studies are still needed to provide more precise picture.

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