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Genetic Polymorphisms of Cyclooxygenase2 (COX2) Gene in Eastern Indian Chronic Periodontitis Patients

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ABSTRACT This present study is aimed to find out the association of Cyclooxygenase2 or COX2 gene polymorphisms such as COX2-765C/G (rs20417), COX2-1195A/G (rs689466) and COX2+8473C/T (rs5275) gene polymorphism with chronic periodontitis in eastern Indian population. A case control study had been performed with a total of 357 participants where 157 identified as patients with chronic periodontitis and the rest 200 were taken as control population. All statistical analysis was performed in SNPassoc, Haploview, MDR 3.0.2 version software packages. The studied COX2 gene polymorphisms are genotypically significantly associated with studied CP population ($\leq$0.01). The mutant alleles of three polymorphisms COX2-765C/G, COX2-1195A/G and COX2+8473C/T are significantly associated to the increased susceptibility of CP (OR= 2.01, 95%CI= 1.475-2.754, p<0.0001; OR= 1.7, 95%CI= 1.249-2.331, p=0.0008; OR= 2.2, 95%CI= 1.61-3.014, p<0.0001 respectively). There are four haplotypes CCA, TGG, TCG and CCG found to be related to the increasing risk of CP. All three COX2 gene polymorphisms are found to be significantly associated with chronic periodontitis increased susceptibility in studied population.

INTRODUCTION

Chronic periodontitis (CP) is a severe chronic oral inflammation manifests gum bleeding, extracellular matrix degradation (ECM), teeth loss (Hajishengallis 2015). The accumulation of profuse amount of microbial plaque at the periodontal pocket increase the gingival inflammation is the prior condition of CP. CP is a multifactorial disease including genetic and environmental factors (Rintakoski et al. 2010). Several studies have confirmed the genetic factors as an important and partial determinant factor of CP (Prakash et al. 2015). Cyclooxygenase2 (COX2) is one of the important genes that is involved in chronic periodontitis progression (Mesa et al. 2012). In human chromosome the location of this gene is 1q31.1 that means it is located on long arm of chromosome number 1. COX2 gene encodes cyclooxygenase (COX) enzyme, is also known as prostaglandin endoperoxide synthetase (PTGS) which converts arachidonic acid to prostaglandins (Pinho et al. 2008). It directly increases prostaglandin in tumor tissues and plays a pivotal role in cell proliferation as well as inflammation at the host tissue (Noguchi et al. 2007; Mahalaxmi et al. 2019). The COX2 gene includes many different polymorphic sites and it is located in the macrophages, fibroblasts and leukocytes. The secretion of COX2 is happened when body is suffering from a certain physical condition like inflammation (Hu et al. 2005). Henceforth, COX2 is playing a pivotal role in tumor progression and inflammation. It generally helps to get relief from inflammation by secreting cyclooxygenase to control the inflammatory condition at the host cell (Wang et al. 2013). But if there any variant or base change occurs in COX2 gene sequence then the whole sequence arrangement getting affected. In this state COX2 gene is acted as inducible agent towards inflammatory condition by releasing proinflammatory cytokines such as interleukin1, interleukin6, tumor necrosis factor alpha (Pu et al. 2009; Upadhyay et al. 2009). Henceforth, irregulated expression of COX2 gene plays key role in influencing the degradation of extracellular matrix (ECM) in periodontal tissue and is supposed to be related to chronic periodontitis susceptibility.

There are three major polymorphisms in COX2 gene which are studied mostly, those are COX2-765C/G (rs20417), COX2-1195A/G (rs689466) and COX2+8473C/T (rs5275). A plenty of research was carried out to find the role of these COX2 gene polymorphisms in CP susceptibility in various population. In Taiwanese pop-

ulation COX2-765C/G gene polymorphism is found to be associated with the decreased susceptibility of chronic periodontitis while Li et al. found a significant association in Chinese population (Ho et al. 2008; Li et al. 2012). Another study was performed by Loo et al. and they reported the positive association of COX2-765C/G gene polymorphism with chronic periodontitis susceptibility (Loo et al. 2011). Another study in Chinese population showed association between COX2-1195A/G gene polymorphism and CP (Xie et al. 2009). In north Indian population, COX2-765C/G gene polymorphism is found to be associated with increased susceptibility of CP only while (Prakash et al. 2015) another study in the same population found that the COX2-1195A/G and COX2+8473C/T gene polymorphisms are not associated to the CP susceptibility (Daing et al. 2012). Schaefer et al. (2010) performed the analysis on COX2-765C/G, COX2-1195A/G polymorphism in German CP patients and found no significant association between them. A recent study has been performed in Iraqi population which revealed positive association COX2-1195A/G polymorphism with increased chronic periodontitis susceptibility (Mahmood et al. 2019).

Objectives

- To find out the association of three COX2 gene polymorphisms; COX2-765C/G (rs20417), COX2-1195A/G (rs689466) and COX2+8473C/T (rs5275) with CP patients from eastern Indian population.
- To study the genotypic and allelic distribution of these above-mentioned gene polymorphisms in CP and control population.
- To find out the haplotype distribution in CP population
- To analyse the allelic combinations associated with increased and decreased susceptibility risk in CP patients

To the best of the researcher's knowledge, in eastern Indian population (mostly Bengal population) this study has been conducted for the first time. There are three important polymorphic sites in the COX2 gene those are COX2-765C/G (rs20417), COX2-1195A/G (rs689466) and COX2+8473C/T (rs5275). These three polymorphisms are located on the promoter region of COX2 gene. The details of studied polymorphism of COX2 gene and functions are discussed.

MATERIAL AND METHODS

Genotyping

The purified genomic DNA was isolated from 357 participants' venous blood. Among 357 participants, 157 were identified as chronic periodontitis patients and the rest 200 were determined as control group after getting their informed consents. The studied subjects were Dr. R. Ahmed Dental College and Hospital based and those were both residents and outpatients. This study was done in accordance with Helsinki declaration and Indian Council of Medical Research guidelines. The whole study was approved by the institutional ethics committee. The selected COX2 genes SNPs, that is, COX2-765C/G (rs20417), COX2-1195A/G (rs689466) and COX2+8473C/T (rs5275) were genotyped through PCR followed by RFLP and Sanger sequencing. All PCR was carried out in 50µl volume. Each reaction volume containing 0.1 µg -0.2µg of DNA, 5µl of 10x buffer, 5µl of 0.5mM MgCl2, 1µl of 10mM dNTPs, 1µl of 0.5 µM of each primer (Metabion®, India), 2U Taq DNA polymerase (Invitrogen®). For RFLP, 1.5U of each restriction enzyme had been used and confirmed them with Sanger sequencing. In Table 1, all designed primers and restriction enzymes along with the conditions have been shown. For selection of restriction enzyme, the researcher's used Gene Runner software and NEBcutter were used to select restriction enzymes. Sequencing was done in ABI Prism 3500 DNA Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Statistical Analysis

All genotypic, allelic distribution for each group were calculated by using 2X2 Chi square (χ^2) test. This 2X2 contingency tables were constructed for Chi square (Pearson Chi square test, likelihood ratio) and Fisher's exact test were performed to justify the statistical significance of genotypic differences between patients and control group. The association analysis of SNP and CP susceptibility was performed under different genetic models by SNPassoc. To justify

Table 1: COX2 genotyping

Gene polymorphism	*Primers (5'-3')*	*PCR condition*	*Restriction enzyme*	*RFLP condition*
COX2-765C/G (rs20417)	F-TGGAAAAGTGGACA GAAAAGACAR-GTGAC GACGCTTAATAGGCTG	1 cycle 94°C for 6min; 25 cycles (94°C for 30sec, 58°C for 1min, 72°C for 1min); final extension at 72°C for 7min	AciI	incubate at 37°C for 18 hrs.489bp (C); 260 bp+229bp (G)
COX2-1195 A/G(rs689466)	F-AGTCATCCGTGTCT CATGAAGAR-CCTGATG CGTGGATTATTTTGG	1 cycle 94°C for 5min; 25 cycles (94°C for 30sec, 56°C for 1min, 72°C for 1min); final extension at 72°C for 7min	PvuII	incubate at 37°C for 18 hrs.549bp (A); 300 bp+249bp (G)
COX2+8473C/T (rs5275)	F-TGTTGCGGAGAAA GGAGTCAR-CCACCACC TTAATAAATTTGGCA	1 cycle 95°C for 5min; 25 cycles (95°C for 30sec, 59°C for 45sec, 72°C for 1min); final extension at 72°C for 7min	BclI	incubate at 37°C for 1 hr.674bp (C); 370bp+304bp (T)

the distribution of genotypic frequencies in studied population Hardy-Weinberg equilibrium test was done with goodness-of-fit χ^2 critical value. The odds ratio (OR) were estimated with 95 percent confidence interval (95% CI) and probability value (p value) of <5 percent was considered to be statistically significant by using medcalc. Bonferroni correction has been made for multiple comparison. The haplotype analysis has been done for linkage analysis to investigate the tendency polymorphisms to be inherited together by using Haploview software. The gene polymorphisms interaction along with their affective allelic combination was measured by Multifactor Dimensionality Reduction (MDR 3.0.2. version) software.

RESULTS

The frequencies of all three genotypes of COX2 in the CP group were found to be in agreement with the Hardy–Weinberg equilibrium ($P>0.001, \chi^2<10.83$). In Figure 1, RFLP outcomes have been shown. All chromatograms of these three COX2 polymorphisms have shown in Figure 2. The genotypic distribution, modelwise genotypic distribution and allelic frequencies of COX2 promoter gene polymorphisms in the CP and HC groups are presented in Table 2, Table 3 and Table 4, respectively. The haplotype distribution of CP population is tabulated in Table 5 and displayed in Figure 3. All data were considered to be significant at 5 percent level of significant. Then all the p values finally considered as significant after Bonferroni's correction which is ≤ 0.01.

Genotypic Distribution

In Table 2 genotypic frequencies in CP in compared to HC population are tabulated. COX2-765C/G polymorphism is significantly associated in CP population. 19.1 percent GG genotype present in CP population which is very high in compared to 8.5 percent in HC population. Hence, this polymorphism is significantly associated with CP risk (OR= 3.93, 95% CI=1.983-7.808, p<0.0001). In COX2-1195A/G, frequency of AG genotype is 49.7 percent in CP and 37 percent in HC group whereas GG frequency in CP is 16.6 and 9.5 percent in HC population. This polymorphism is also related to increasing susceptibility to CP (OR= 2.762, 95% CI= 1.403-5.436, p= 0.003). CC genotype frequency of third COX2 polymorphism in CP is 28.7 percent and 51.5 percent in HC. CT genotype frequency is 52.2 percent in CP and 38.5 percent in HC. TT frequency in CP is 19.1 percent and in HC is 10 percent. Hence, COX2+8473C/T polymorphism is also significant towards the increased susceptibility to CP prevalence (OR= 3.433, 95%CI= 1.764-6.679, p=0.0002). Henceforth, all three studied COX2

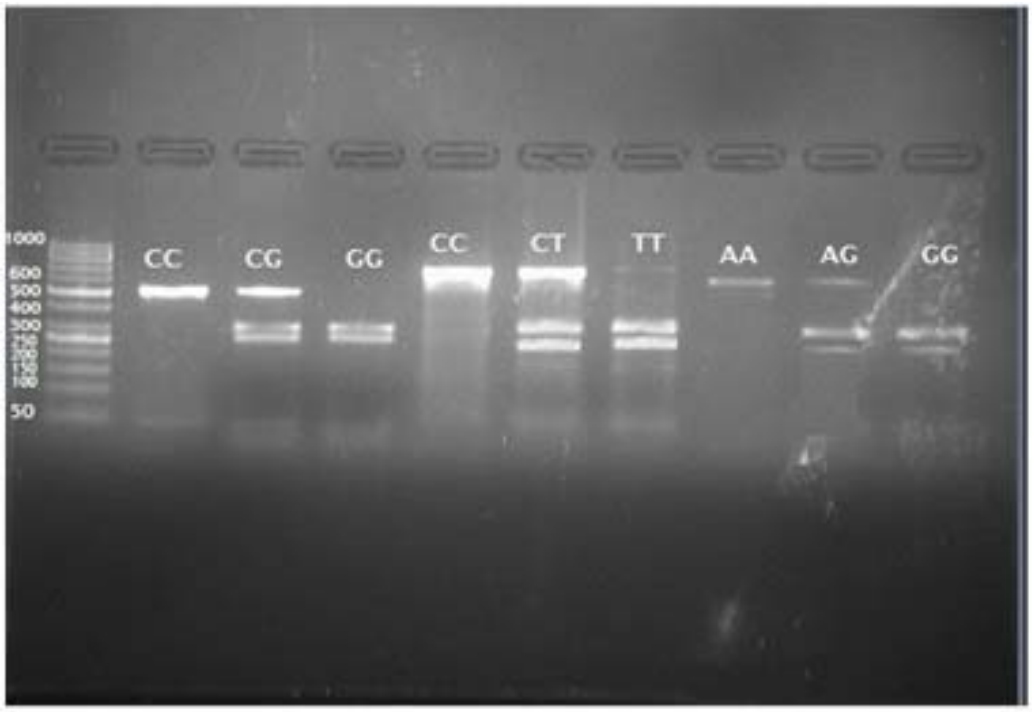

Fig. 1. RFLP of COX2-765C/G, COX2+8473C/T and COX2-1195A/G polymorphism
Source: Author

Table 2: Genotypic frequency in CP and HC population

Cox2 gene SNPs(rs number)	*Geno-type*	*CP(%)*	*HC(%)*	*χ^2 (p-value)*	*OR (95% CI)*	*Fisher exact P value*
-765C/G	CC	48 (30.6)	107 (53.5)			
(rs20417)	CG	79 (50.3)	76 (38.0)	12.82 (0.003)	2.317(1.457-3.684)	<0.0001*
	GG	30 (19.1)	17 (8.5)	16.43 (<0.0001)	3.93 (1.983-7.808)	<0.0001*
-1195A/G	AA	53 (33.8)	107 (53.5)			
(rs689466)	AG	78 (49.7)	74 (37.0)	9.86 (0.001)	2.12 (1.346-3.363)	0.001*
	GG	26 (16.6)	19 (9.5)	9.01 (0.002)	2.762 (1.403-5.436)	0.003*
+8473C/T	CC	45 (28.7)	103 (51.5)			
(rs5275)	CT	82 (52.2)	77 (38.5)	14.16 (0.0001)	2.437 (1.525-3.893)	0.0002*
	TT	30 (19.1)	20 (10.0)	13.91 (0.0001)	3.433 (1.764-6.679)	0.0002*

*Statistically significant ≤0.01.

gene polymorphisms are seemed to be associated with increased susceptibility to CP occurrence.

Model Wise Distribution

The researcher has discussed four different models for genotypic distribution (Table 3) of COX2 gene polymorphisms in CP population. Though genotypic distribution of COX2-765C/G polymorphisms are significant in CP risk but the model wise genotypic distribution did not show statistical difference between CP and HC (Bonferroni correction ≤0.01). COX-1195A/G polymorphism also showing increased susceptibility to CP risk (Co-dominant model: AA vs

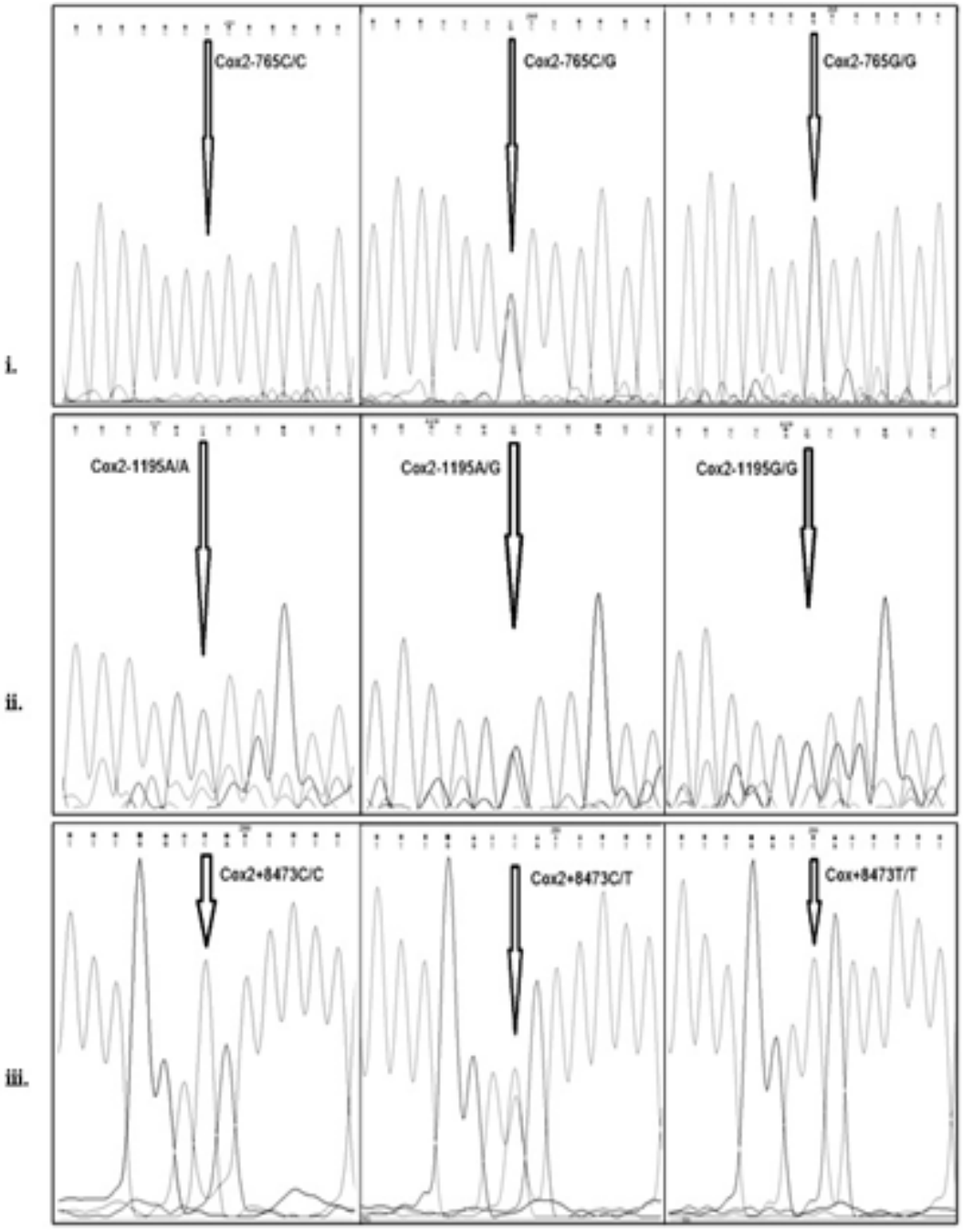

Fig. 2. Chromatograms of i. COX2-765C/G; ii. COX2-1195A/G; iii. COX2+8473C/T
Source: Author

Table 3: Modelwise genotypic distribution in CP and HC population

Cox2 Gene	*Model*		*AOR*	*95% CI*	*p-value*
-765C/G(rs20417)	Co-dominant	CC vs CG	1.57	0.95-2.61	0.154
		CC vs GG	1.71	0.81-3.61	
	Dominant	CC vs CG+GG	1.60	0.99-2.59	0.05
	Recessive	CC+CG vs GG	1.35	0.67-2.72	0.403
	Over dominant	CC+GG vs CG	1.38	0.86-2.21	0.184
-1195A/G(rs689466)	Co-dominant	AA vs AG	2.46	1.46-4.15	0.0005*
		AA vs GG	3.01	1.45-6.26	
	Dominant	AA vs AG+GG	2.59	1.58-4.23	0.0001*
	Recessive	AA+AG vs GG	1.86	0.95-3.64	0.067
	Over dominant	AA+GG vs AG	1.82	1.13-2.93	0.0125*
+8473C/T(rs5275)	Co-dominant	CC vs CT	2.07	1.24-3.45	0.003*
		CC vs TT	2.79	1.31-5.92	
	Dominant	CC vs CT+TT	2.21	1.36-3.59	0.001*
	Recessive	CC+CT vs TT	1.91	0.94-3.86	0.068
	Over dominant	CC+TT vs CT	1.60	1.00-2.57	0.5

*Statistically significant ≤0.01

Table 4: Allelic distribution of COX2 polymorphisms in CP population

Cox2 gene SNPs(rs number)	*Allele*	*CP(%) N=157*	*HC(%) N=200*	*χ^2 (p-value)*	*OR (95% CI)*	*Fisher exact P value*
-765C/G	C allele	176 (0.56)	288 (0.72)			
(rs20417)	G allele	138 (0.44)	112 (0.28)	19.67 (<0.0001)	2.01 (1.475-2.754)	<0.0001*
-1195A/G	A allele	185 (0.59)	284 (0.71)			
(rs689466)	G allele	129 (0.41)	116 (0.29)	11.39 (0.0007)	1.7 (1.249-2.331)	0.0008*
+8473C/T	C allele	173 (0.55)	292 (0.73)			
(rs5275)	T allele	141 (0.45)	108 (0.27)	24.83 (<0.0001)	2.2 (1.61-3.014)	<0.0001*

*Statistically significant ≤0.01

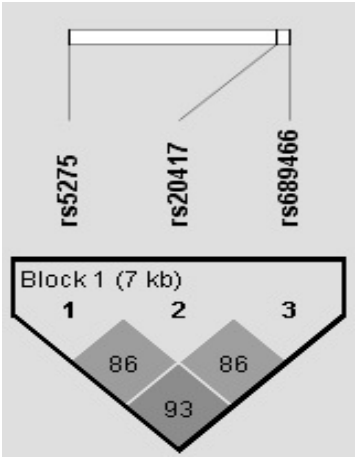

Fig. 3. Haplotype distribution of COX2 gene polymorphisms -765C/G (rs20417), -1195A/G (rs689466), +8473C/T (rs5275) in CP
Source: Author

Table 5: Haplotype distribution in CP

Haplo-types	*Frequencies*	*χ^2*	*P-value*
CCA*	0.602	28.27	<0.0001
TGG*	0.301	10.67	0.001
TCG*	0.033	4.413	0.035
CGA	0.021	0.891	0.345
CCG*	0.020	6.471	0.011

*Statistically significant <0.05

AG; AOR= 2.46, 95%CI= 1.46-4.15, AA vs GG; AOR= 3.01, 95%CI= 1.45-6.26, p=0.0005. Dominant model: AA vs AG+GG; AOR= 2.59, 95%CI= 1.58-4.23, p= 0.0001. Recessive model: AA+AG vs GG; AOR= 1.86, 95%CI= 0.95-3.64, p= 0.067. Over dominant model: AA+GG vs AG; AOR= 1.82, 95%CI= 1.13-2.93, p=0.5). The third one is COX2+8473C/T polymorphism which is also significantly related to the CP risk (Co-dominant model: CC vs CT; AOR= 2.07, 95%CI= 1.24-3.45,

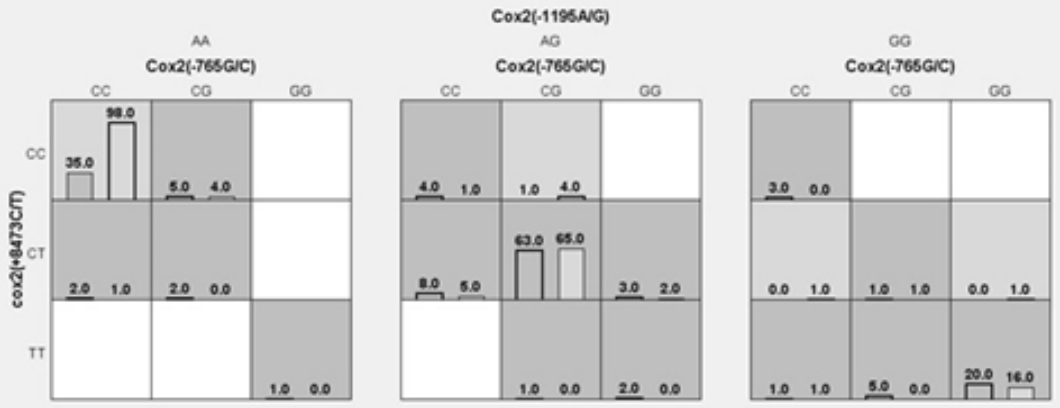

Fig. 4. Allele combinations of indicated COX2 SNPs
Footnote: The SNPs associated with high (dark-grey cells) and low (light-grey cells) susceptibility risk in CP patients. The figure was generated by MDRv.3.0.2 (Computational Genetics Laboratory; Dartmouth).
Source: Author

CC vs TT; AOR= 2.79, 95%CI= 1.31-5.92, p=0.003. Dominant model: CC vs CT+TT; AOR= 2.21, 95%CI= 1.36-3.59, p=0.001. Recessive model: CC+CT vs TT; AOR= 1.91, 95%CI= 0.94-3.86, p= 0.068. Over dominant model: CC+TT vs CT; AOR= 1.60, 95%CI= 1.00-2.57, p=0.5) if consider codominant and dominant model.

Allelic Distribution

The Allelic Distribution of COX2 Gene: In Table 4, allelic distributions were discussed. G allele of COX2-765C/G polymorphism is significantly associated with CP (OR= 2.01, 95%CI= 1.475-2.754, p<0.0001). The number of this G allele in CP is 138 in compared to 112 in control (HC) subjects. Another COX2 polymorphism, -1195A/G, the rare allele, G allele is also seemed to be associated in CP progression (OR= 1.7, 95%CI= 1.249-2.331, p=0.0008). The T allele of +8473C/T polymorphism is associated to decreased susceptibility to CP (OR= 2.2, 95%CI= 1.61-3.014, p<0.0001). The number of T allele in CP population is 141 (45%) and in HC population is 108 (27%). Henceforth, the association of all three COX2 gene polymorphisms' rare alleles are significant with CP increased susceptibility.

Haplotype Distribution: In Figure 3, haploblock of COX2 three polymorphisms in CP population are described. COX2-765C/G (rs20417) polymorphism, -1195A/G (rs689466) polymorphism, +8473C/T (rs5275) polymorphisms are analyzed for linkage disequilibrium analysis. Those three COX2 polymorphisms are linked with each other (d>0.8). Four haplotypes are significantly associated with CP increased risk. Those are CCA, TGG, TCG and CCG haplotypes (p<0.05). In Figure 4, the allelic combination of studied polymorphisms has been featured. The high-risk cells were indicated in dark grey colour where the light grey coloured cells indicating the low-risk zone towards CP susceptibility.

DISCUSSION

The differences in periodontitis among individuals could not be interpreted by differences in oral hygiene alone. The genetic factors are also involved including COX2 gene polymorphisms (Sorsa et al. 2004). The findings of this study are as follows:

- Three studied COX2 gene polymorphisms, that is, COX2-765C/G (rs20417), COX2-1195A/G (rs689466) and COX2+8473C/T (rs5275) are genotypically significantly associated with studied CP population.
- Though model wise genotypic distribution shows the alliance of COX2-1195A/G (rs689466) and COX2+8473C/T (rs5275) gene polymorphism in patient group.
- The allelic distribution of three gene polymorphisms are significantly associated to the increased susceptibility of CP.

- There are four haplotypes CCA, TGG, TCG and CCG found to be related to the increasing risk of CP.
- The most affective allelic combination of these three studied polymorphisms are GG (COX2-765C/G) X GG (COX2-1195A/G) X TT (COX2+8473C/T).

Hence the present study shows statistically significant association of three COX2 gene polymorphisms with CP group in eastern Indian population. All those polymorphisms are associated with the increased susceptibility to CP. In -765C/G polymorphism, the substitution of G instead of C causes effect on transcription of coded protein. If this base change occurs the changes in protein code getting affected and it becomes unable to prevent inflammation and indirectly helps in chronic periodontitis progression (Prakash et al. 2015). The same concepts are laying on those remaining polymorphisms. However, the concept of association of SNPs is still not understandable. This work helps in finding the geno-etiology towards the association between COX-2 gene polymorphism and periodontitis risk. There are some different studies performed in Asian population and the outcomes are different from each other (Zhang et al. 2018). This may happen due to the genetic diversity throughout population. In addition, the studied population is obeying HWE law though a large number of population study is needed. The haplotype distribution is also showing positive alliance with increased susceptibility to CP. The linkage frequencies of COX2+8473C/T with COX2-765C/G is 86 percent and with COX2-1195A/G is 93 percent. The linkage frequency between COX2-765C/G and COX2-1195A/G is 86 percent. If the linkage frequency if more than 80 percent ((d>0.8) it is thought that those significant haplotypes are prone to transmit in next generation and may increase the risk of CP disease. While working on gene polymorphisms interactions on CP progression, a very allelic combinations have been found to be affective. The most high-risk cells have been found in the second and third interaction block which are fourteenth and twenty-seventh cells respectively. The most affective allelic combination is comprised of all variant allele of these three gene polymorphisms, that is, GG (COX2-765C/G) X GG (COX2-1195A/G) X TT (COX2+8473C/T) (twenty-seventh cell) while the second most affective combinations, that is, CG (COX2-765C/G) X AG (COX2-1195A/G) X CT (COX2+8473C/T) (fourteenth cell). On the contrary, the low risk allelic combination is comprised of all wild type alleles of studied polymorphisms.

This study is purely focused on gene polymorphisms and their different kind of distribution along with combination which many affect the CP progression in studied population. As earlier mentioned in this study that not only the genetic factors but also the clinical and environmental factors may involve in this disease progression due to its multifactorial nature. Clinical factors include the periodontal features such as pocket depth (PD), clinical attachment loss (CAL), bleeding on probe (BOP), oral hygiene, number of teeth loss etc. on the other hand, the environmental factors mostly focused on basal characteristics of studied population such as age, gender, oral habits like smoking, chewing tobacco (Albandar et al. 2002; Majumder et al. 2019c). That clinical study along with basal characteristics have been performed in the same population in previous study (Majumder et al. 2019b). There are also different gene polymorphism studies have been performed in this population which have shown different kind of outcomes though the study on COX2 gene polymorphisms has been performed first time (Majumder et al. 2017, 2018, 2019a).

CONCLUSION

This study concludes the significant association of COX2 gene polymorphisms COX2-765C/G (rs20417), COX2-1195A/G (rs689466) and COX2+8473C/T (rs5275) with CP in eastern Indian population. There are four haplotypes CCA, TGG, TCG and CCG, which are found to be associated with the increased susceptibility of CP. This study also infers the allelic distribution of studied three gene polymorphisms, those are significantly associated to the increased susceptibility of CP.

RECOMMENDATIONS

According to the findings, a large number of study population would be required to make this work more enriched and profound. Further stud-

ies related to protein expression of studied COX2 genes and their impact on chronic periodontitis progression should be performed in future.

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ABBREVIATIONS

A: Adenine
C: Cytosine
G: Guanine
T: Thymine
COX2: Cyclooxygenase2
CP: Chronic Periodontitis
MDR: Multidiamensional Resonance
SNP: Single Nucleotide Polymorphism
PD: Pocket Depth
CAL: Clinical Attachment Loss
BOP: Bleeding on Probe
PTGS: Prostaglandin Endoperoxide Synthetase
DNA: Deoxyribonucleic Acid
PCR: Polymerase Chain Reaction
RFLP: Restriction Length Polymerase

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