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"ASSOCIATION OF CXCL8 -251 T>A GENE POLYMORPHISM IN DIABETIC NEPHROPATHY AND CARDIOVASCULAR DISEASE IN WEST INDIAN POPULATIONS"

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ABSTRACT
Type 2 diabetes mellitus (T2DM) is one of the most important global public health problems which is rapidly increasing and it is considered an epidemic disorder worldwide.CXCL8 Chemokine (C-X-C motif) has been recognized as a central player in type 2 diabetes mellitus, diabetic nephropathy and cardiovascular disease. The aim was to find the relationship between CXCL8 -251 A>T polymorphisms and their association with DN, cardiovascular disease (CVD) in type 2 diabetic patients. The population consisted of 631 participants (458 males and 173 females) allocated into six groups for the study. The first group was healthy participants (n=143); the second group was T2DM patients (DM without any complication, n=103); the third group diabetic nephropathy with diabetes (DN+DM, n=102). The fourth group was non diabetic nephropathy (NDN-DM, n=108) the fifth group was cardiovascular patient with diabetes (CVD+DM, n=81) and the sixth group was non diabetic cardiovascular (NDCV-DM, n=94). The polymorphism were analyzed by PCR-RFLP using *Mfe*I restriction enzymes which was checked on 3% agarose gen consisting of ethidium bromide for detection of restricted band. The aim of the present study was to investigate the association between CXCL8 gene polymorphisms (-251 T>A) polymorphism in West Indian population. This study is aimed to evaluating the association of CXCL-8 gene polymorphisms (-251 T>A) with complicated and uncomplicated type 2 diabetes mellitus. AT genotype are higher in T2DM, DN and CVD patients compared with control (P<0.05), In addition, the T allele of CXCL8 -251 T>A gene are more susceptible to DN and CVD.

KEYWORDS: CXCL8, PCR-RFLP, T2DM, Diabetic nephropathy, Genotypic and allelic frequency.

INTRODUCTION
Diabetes is the most common endocrine disorder, affecting in the year 2013, about 382 million people worldwide and it is estimated that in 2035, over 592 million cases of diabetes will be registered (Diabetes FD 2013). Diabetic nephropathy, is a chronic, progressive kidney disease and represents a complication of type 1 or 2 diabetes, being the first cause of terminal chronic kidney failure and affects 30-40% of patients with diabetes (Diabetes FD 2013). In the scientific literature, many recent researches present the diversity of risk factors, the multitude of cellular and molecular alterations in diabetic nephropathy, estimated factors that could prevent or ameliorate complications associated with diabetes.[1],[2],[3],[4],[5] It is considered that chemokines play an important role in various inflammatory circumstances. In the last years, evidence was gained regarding the intervention of CXCL8 in glomerular architectural conservation, within the modulation mechanisms of the renal response to various internal and external insults.[6],[7],[8]

CXCL8 is one of the first and most intensively studied chemokines acting as a pro-inflammatory chemokine. In the late 1980s, Peveri et al. found that LPS-stimulated blood monocytes produced a secretory protein (neutrophil activating factor, NAF) that stimulated neutrophil exocytosis (granule release) and oxidative burst (superoxide and hydrogen peroxide production) that appeared to be mediated by cell surface receptors.[9] NAF was the first chemokine to be purified and sequenced in 1987 and was later named as CXCL8 upon identification of additional chemokines.[10],[11],[12] The crystal structure of CXCL8 was first solved in 1991. CXCL8 is a 72-aa length peptide has three antiparallel β-strands and one α-helix made up of C-terminal residues

57-72.[13] The structure is stabilized by two disulfide bonds formed by Cys7-Cys34 and Cys9-Cys50. The two cysteine residues (Cys7 and Cys9) are separated by one residue (Gln8), hence CXCL8 is classified as CXC chemokine. The crystal structure also revealed that CXCL8 exists as a dimer stabilized by hydrogen bonds between the first β-strand.

CXCL8 has been described as a chemokine participating in all stages of atherosclerosis from vascular inflammation to cardiac remodeling after myocardial infarction (MI).[14] CXCL8 promotes and sustains the shift from an acute to a chronic inflammatory reaction in the vessel wall by mediating the release of the monocyte chemo-attractant protein 1 (MCP-1) and the shedding of soluble receptor of the interleukin 6 (IL6) from the neutrophils in the inflammatory infiltrate.[15] The association of CXCL8 serum levels and of CXCL8 gene (IL8) with the risk of coronary heart disease (CHD) has been investigated in a few small studies with contradictory findings. It has become apparent that inflammatory mechanisms contribute expressively to the advance and progression of DN. These comprise the permeation of renal compartments by lymphocytes and monocytes/macrophages as well as local manufacture of cytokines and chemokines in the kidney.[16],[17] The aims of this study were to examine the association of CXCL8 -251 T>A polymorphism with the patients having T2DM and its complication.

MATERIAL AND METHODS

Participants

The population consisted of 631 participants (458 males and 173 females) allocated into six groups for the study. The first group was healthy participants (n=143); the second group was T2DM patients (DM without any complication, n=103); the third group diabetic nephropathy with diabetes (DN+DM, n=102). The fourth group was non diabetic nephropathy (NDN-DM, n=108) the fifth group was cardiovascular patient with diabetes (CVD+DM, n=81) and the sixth group was non diabetic cardiovascular (NDCV-DM, n=94). Proper informed consent was taken from all participants and the study was permitted by the ethics committee (approval no-HMPCMCE: HREC/FCT/47/9 of H. M Patel Center for Medical Care and Education Karamsad, Anand, Gujarat.

Genotyping

Genomic DNA from and controls cases and different groups was extracted from peripheral blood leukocytes by proteinase K/chloroform/isopropanol treatment.[18] The purified DNA was used to determine the genotypes of both polymorphisms, using polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) methods. The PCR primers for CXCL8 -251 T>A polymorphism were 5'-TTCTAACACCTGC CACTCTAG-3' (sense) and 5'-CTGAAGCTCCACAAT TTGGTG-3' (antisense), A 30 µl reaction mixture contained 1x PCR buffer, 1.5 mmol/l MgCl2, 1 µmol/L each primer, 0.2 mmol/L each deoxynucleoside triphosphate (dNTP), 50 mL/L formamide, 2.0 U Taq polymerase, and 25 ng genomic DNA. The thermal cycling conditions included an initial denaturing at 94°C for 5 minutes, followed by a 35 cycles of denaturing at 94°C for 30 seconds, annealing at 61.5°C for 30 seconds, and extension at 72°C for 30 seconds. A final elongation step of 72°C for 5 minutes was included. After confirmation of an amplified fragment of the expected size 108bp on 2% agarose gel, 8-12 µL of PCR products were digested overnight at 37°C with the appropriate restriction enzymes. *MfeI* (New England Biolabs) were used, the digested fragments were resolved on 3% agarose gel to check the polymorphism for CXCL8 -251 T>A containing ethidium bromide (**figure 1**). The wild homozygous alleles (T/T) yielded a108 bp product, and the heterozygous alleles (T/A) yielded 108, 76, and32bp products, while the mutant homozygous alleles (A/A) yielded 76 and 32bp products.

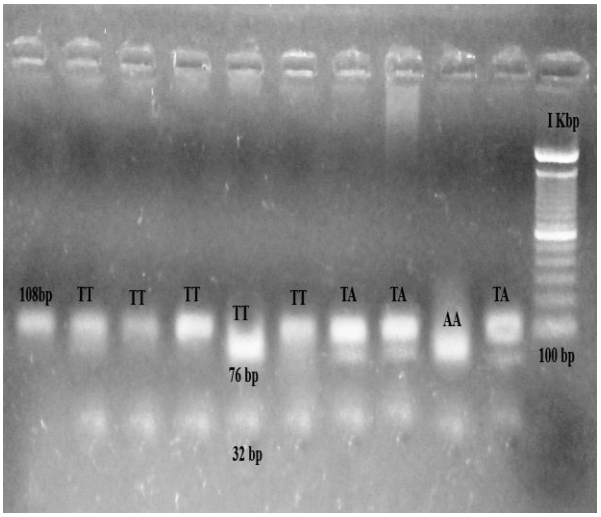

Fig. 1: 3% Agarose gels electrophoresis of CXCL-8 gene showing polymorphism stained with ethidium bromide. The wild homozygous alleles (TT) yielded a 108-bp product, and the heterozygous alleles (TA) yielded 108, 76, and 32 bp products, while the mutant homozygous alleles (AA) yielded 76 and 32 bp products.

STATISTICAL ANALYSIS

The SPSS package system version 14.0 for windows was used for data management and analysis. Quantitative data was presented as median and mean ± standard deviation (SD). Qualitative data was expressed as frequency (absolute numbers and percent %). Pearson chi-square and Fisher's exact tests were used to compare between independent proportions. Statistical analysis considered to be significant when *p*-value was 0.05. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to estimate the strength of the association.

RESULT AND DISCUSSION

A significant association of allele 'A' of CXCL-8 -251 T>A polymorphism with different groups was observed. The analysis shows a significant A allele in DN+DM

(p=0.008, $\chi2$=7.424, OR=0.590, 95% CI=0.396-0.879), CVD+DM (p=0.020, $\chi2$=5.726, OR=0.609, 95% CI=0.396-0.933) when compared to control groups, NDN-DM (p=0.003, $\chi2$=9.012, OR=1.849, 95% CI=1.212-2.824) compared to DN+DM and CVD+DM (p=0.008, $\chi2$=7.183, OR=0.558, 95% CI=0.355-0.875) when it was compared to NDN-DM.

A significant association of the heterozygous genotype TA was observed in CVD+DM (p=0.001, $\chi2$=10.239, OR=5.117, 95% CI=1.622-17.945) when compared with the control groups, CVD+DM (p=0.003, $\chi2$=8.885, OR=5.004, 95% CI=1.477-18.638) when compared with the DM groups and CVD+DM (p=0.004, $\chi2$=8.779, OR=4.843, 95% CI=1.463-17.689) when compared with the DN+DM groups.

A significant association of the homozygous genotype AA was also observed in DM (p=0.001, $\chi2$=11.618, OR=4.555, 95% CI=1.707-12.391) when compared with control groups, CVD+DM (p=0.001, $\chi2$=10.868, OR=7.615, 95% CI=1.843-34.153) and NDCV-DM (p=0.013, $\chi2$=6.707, OR=0.292, 95% CI=0.100-0.840) when compared with the DN+DM groups (**table 1**).

Table 1: Distribution of genotype and allele frequencies of the CXCL-8 -251 T>A polymorphism in Type 2 diabetes (DM), diabetic nephropathy with diabetes (DM+DN), type 2 diabetes without nephropathy (DM−DN), cardiovascular with diabetes (CV+DM), cardiovascular without diabetes (CV−DM) and Healthy controls showing significant Odd ratio (OR), 95% Confidence Interval (CI), chi-square value and p value ($p \leq 0.05$) when compared with each group.

CXCL-8 -251 T>A Polymorphism	Control (n=143)	DM (n=103)	DN + DM (n=102)	NDN – DM (n=108)	CVD + DM (n=81)	NDCV – DM (n=94)
Genotype frequencies (N= %)						
TT	34 (23.77)	19 (18.44)	22 (21.56)	65 (60.18)	4 (4.93)	58 (61.70)
TA	98 (64.53)	56 (54.36)	67 (65.68)	23 (21.29)	59 (72.82)	26 (27.65)
AA	11 (7.69)	28 (27.18)	13 (12.74)	20 (18.51)	18 (22.22)	10 (10.63)
Allelic frequencies (N= %)						
	(2n=286)	**(2n=206)**	**(2n=204)**	**(2n=216)**	**(2n=162)**	**(2n=188)**
T	165 (57.69)	132 (64.07)	141 (69.11)	120 (55.55)	112 (69.13)	118 (62.76)
A	121 (42.30)	74 (35.92)	61 (29.90)	96 (44.44)	50 (30.86)	70 (37.23)
Allele (P value, Chi square, OR and 95% CI)						
		Cont. vs DN+DM	**Cont. vs CVD+DM**	**DN+DM vs NDN-DM**	**NDN-DM vs CVD+DM**	
A allele		P=0.008 7.424 0.590 (0.396-0.879)	P=0.020 5.726 0.609 (0.396-0.933)	P=0.003 9.012 1.849 (1.212-2.824)	P=0.008 7.183 0.558 (0.355-0.875)	
Genotype (P value, Chi square, OR and 95% CI)						
TA (Heterozygous)				**AA (Homozygous)**		
Cont. vs CVD+DM	**DM vs CVD+DM**	**DN+DM vs CVD+DM**		**Cont. vs DM**	**DN+DM vs CVD+DM**	**DN+DM vs NDCV-DM**
0.001 10.239 5.117 1.622-17.945	0.003 8.885 5.004 1.477-18.638	0.004 8.779 4.843 1.463-17.689		0.001 11.618 4.555 1.707-12.391	0.001 10.868 7.615 1.843-34.153	0.013 6.707 0.292 0.100-0.840

The approximately 50 known human chemokines are grouped into four families on the basis of conserved cysteine residues near their N-terminus, and are designated as the CC, CXC, C, and CX3C subfamilies. The CXC chemokine ligands are further subdivided on the basis of the presence or absence of another three-amino acid sequence, glutamic acid–leucine–arginine (the ELR sequence motif), immediately proximal to the CXC sequence.[19] The ELR positive CXC chemokines, which include interleukin-8 (CXCL8), are potent neutrophil chemoattractants. The chemokine-receptor binding initiates a complex signaling cascade that generates chemotactic responses, degranulation, release of ROS and changes in the affinity of cell surface integrins.[20]

Several polymorphisms in a given individual may contribute to the individual risk of developing the disease. In our study, presence the of CXCL8 -251 A allele was more frequent in patients than in controls, suggesting an increased risk for the disease. Whereas TA genotype were significantly more frequent in healthy controls suggesting a protective effect.

The purpose of our study was to assess the role of the CXCL8 polymorphisms as the risk factors for DN and CVD in a case-control setting. There is very little

research considering the association between the CXCL8 -251 T/A polymorphism and DN and CVD. It was reported that the presence of the -251 A allele affects the expression of the CXCL-8 gene which also confirms our studies in both DN and CVD.[21] Further studies, including promoter assays, confirmed the functional role of the CXCL8 -251 T/A T/A polymorphism.[22][23]

The presence of the CXCL8 -251 T/A polymorphism in the transcription start site has been associated with various diseases; however, results of different studies are often contradictory. In our outcome, we have found that an association between the AA genotype and A allele and the risk of DN and CVD. It was also found that CXCL8 -251 AA genotype to be associated with higher risk of DN in Asians Indians population which confirms our study.[2] The CXCL8 T-251 A variant lies in the regulatory region and increases the gene expression suggesting that this genotype could regulate CXCL8 gene expression.[22] Increased urinary excretion of CXCL8 has been reported in DN patients.[8] This variant has been associated with inflammatory renal injury.[24] Our results are also in line with the outcomes of the study performed in the American population which showed that carriers of the CXCL8 -251 A allele were at a 1.5-fold increased risk of colon adenoma.[25] This research also indicates that the TA heterozygote may also increase the risk of CVD occurrence. This has been confirmed by Lurje et al. who revealed that American patients carrying the AA genotype were at a higher risk of developing tumor recurrence.[26] The study showing an association of this allele with increased risk of CVD had not been carried out by others so much more work has to be carried out to confirm.

CONCLUSIONS

CXCL8 -251 T/A plays an important role in the development of renal response to various internal and external insults, by recruiting neutrophils, basophils and T lymphocytes in the affected areas of the kidney. We observed an association between the TA heterozygote genotype and A allele of the CXCL8 T>A polymorphism and CVD, although the study also signifies the association of AA homozygotes in the progression of CVD but to a lesser extent, the presence of CXCL8 T>A A allele were significant associated with the development of both DN and CVD. Our work revealed that the polymorphism tested of the CXCL8 gene suggest a shielding effect against DN and CVD in the West Indian ethnic population. Whereas this finding is potentially important, but it requires further ratification in larger other populations.

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