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Case Control Study

Association of *NFKB1* gene polymorphism (rs28362491) with levels of inflammatory biomarkers and susceptibility to diabetic nephropathy in Asian Indians

Reviewer (ID: 00503187)

Comment 1): In abstract in aims, mention the name of the gene that is studied.

Answer to comment 1): The name of the gene has been mentioned as suggested on Page 3, Line no: 2

Comment 2): In the first sentence of discussion (NFKB1 promoter (-94 ins/del) AGGT polymorphism has been associated with many inflammatory diseases like asthma, autoimmune diseases like rheumatoid arthritis, cancers, AIDS, and various diabetic complications.), please, give references for previously published work. Do the 'various diabetic complications' include studies on diabetic nephropathy?

Answer to comment 2): Reference has been incorporated in the manuscript as per reviewer's suggestion on Page 11, Line no: 4. Various diabetic complications include atherosclerosis as well as nephropathy in European population.

Comment 3): The last sentence of the first paragraph in discussion (However our results were in contrast with a genomic study conducted by Yang et al in 2014[28].) leaves open the reasons for the contradictory results. The authors could discuss the reasons for the contradictory findings.

Answer to comment 3): The discussion for the contradictory findings is mentioned in the discussion section on Page 11, Line no: 18-20.

Comment 4): I suggest the authors to modify the sentence in discussion saying 'A comparable study showed that p50 null mice...'. The comparison is done between a study on mice and a study on patients with T2DM; I don't think that these two setups can really be called comparable. Also, the comparison is made between development of nephropathy and asthma, arthritis, and autoimmune encephalomyelitis.

Answer to comment 4): The sentence in discussion saying 'A comparable study showed that p50 null mice...'. has been modified as per suggestion (Page no 12, Line no:15-18).

Comment 5): Also the next sentence ' A similar study conducted in sporadic colorectal cancer (CRC)[33] and epithelial ovarian cancer (EOC)[26]' needs modification. It is called 'similar study'. On what basis are the studies similar? The sentence could be modified.

Answer to comment 5): Study conducted in sporadic colorectal cancer (CRC)[33] and epithelial ovarian cancer (EOC)[26] can be considered similar to our study since inflammation plays an important role in the pathogenesis of the mentioned cancer. It is also known fact that diabetes and nephropathy is considered currently as inflammatory disease. Mohd Suzairi et al, Huo et al and as well we have tried to investigate the association of NFKB1 -94 ins/del AGGT polymorphism with CRC, EOC and diabetic nephropathy.

Comment 6): The language needs to be improved throughout the manuscript.

Answer to comment 6): The language is improves as suggested by reviewers.

Reviewer (ID: 02533652)

Comment: The manuscript is well informative, however it is based on small sample size which is a limitation of generalising the conclusion. Discussion is also weakly written and there are too many typos and grammatical mistake all across the manuscript. It is advisable to revamp the discussion and add more comparisons to the other studies in more detailed fashion

Answer: We are very thankful for your valuable suggestion to improve our manuscript. We would like to bring your kind notice that we have already mentioned about the small sample size of our current study as a limitation on Page no 13, Line no: 6-7. However, we have now clearly mentioned about the limitation of our study in conclusion section, Page no 13, Line no: 12-15.

As per reviewer's suggestion, we have reframed the discussion section of the manuscript. We have also checked the entire manuscript for typos & grammatical mistakes and corrections have been made as per suggestions.

Reviewer (ID: 03490249)

Gautam A et al. investigated -94 ATTG insertion/deletion polymorphism in NFKB1 gene in normoglycemic, type 2 diabetes without complications, and with diabetic nephropathy. The allelic frequencies of -94 ATTG insertion/deletion were 0.655/0.345 (NG), 0.62/0.38 (DM) and 0.775/0.225 (DM-CKD). The -94 ATTG ins allele was associated with elevation of urinary excretion of uMCP-1, and plasma TNF-alpha. The authors demonstrated that -94 ATTG ins allele is risk for the development of diabetic nephropathy. However, they should carefully avoid the selection bias in the case control study

Major comments

Comments 1. The authors should mention about the ethnic differences.

Answer to Comment 1. As per reviewer's suggestion ethnic differences have been incorporated in manuscript on Page no 11, Line no: 9-17.

Comments 2. The authors should define ‘normoglycemic’ in page 7 line 16. Is it normal fasting glucose or normal glucose tolerance? Did authors perform 75g-OGTT?

Answer to Comment 2. We have enrolled subjects with normoglycemia purely on basis of diagnosis by American Diabetic association 2015 criterion. Subjects is normoglycemic when his/her fasting plasma glucose < 100 mg% or postprandial glucose < 140 mg% or HbA1c <5.7%. Definition of normoglycemia is incorporated in the manuscript as per suggestion on Page no 7, Line no:3-4. We did not perform 75g-OGTT.

Comments 3. Why did authors define ‘DM-CKD’ as subjects with T2DM > 5 years with nephropathy? The patients with T2D develop diabetic nephropathy in 5 years?

Answer to Comment 3. This difference of duration for T2DM and DN groups was intentionally chosen as our inclusion criteria. Many patients suffering from T2DM ≥10 years do not develop any microvascular and macrovascular complication,thus ruling out the genetic risk factors. However, it has been seen that in spite of shorter duration of T2DM i.e. ≥5 years patients may develop microvascular complications like DN. Therefore we wanted to explore the risk factors behind the observation that why diabetic patients of small duration of 5 years develop nephropathy and patients having diabetes for greater than 10 years do not develop complication particularly nephropathy.

Comments 4. In page 7 line 27, how did authors measure the proteinuria and microalbuminuria?

Answer to Comment 4. We are very grateful for the valuable comment. But I would like to bring you kind notice that we have already mentioned the method of estimation of microalbuminuria by urinary dipstick(Urine Test 11 MAU, Piramal Diagnostic). However,

the sensitivity of the method has now been incorporated in the manuscript on Page no 6, Line no: 29. However, it was further confirmed by urinary albumin to creatinine ratio which is measured by colorimetric method using commercially available kits on an autoanalyser Olympus AU-400.

Comments 5. In page 8 lines 3-4, how did authors check the presence of diabetes in the relatives?

Answer to Comment 5. The presence or absence of diabetes mellitus in the relatives of the subjects was checked by taking detailed medical history. Relatives who gave negative history of diabetes where further confirmed by estimating fasting plasma, postprandial glucose and HbA1c.With fasting plasma glucose < 100 mg% or postprandial glucose < 140 mg% or HbA1c <5.7% were considered non diabetic or normoglycemic.

Comments 6. In page 8 line 7, please specify 'renal disorders'.

Answer to Comment 6. As per reviewer's suggestion, we have specified 'renal disorder' in the manuscript on Page 7, Line no: 9-10.

Comments 7. In page 8 line 10, why did authors eliminate the patients with macrovascular complications? How did authors check the presence of macroangiopathy?

Answer to Comment 7. Chronic inflammation is a risk factor for macrovascular complication like stroke and coronary artery diseases (Willerson JT et al, DOI: 10.1161/01.CIR.0000129535.04194.38; Wang Q et al, PMID: 17901552) . However, microvascular complication like nephropathy is also due to long standing low grade inflammation. However, we mainly focused on association of nephropathy and inflammatory markers. If we would have not eliminated macrovascular complications then we would have not able to measure true levels of plasma inflammatory markers causing nephropathy. Authors checked for the presence of macroangiopathy by medical history of chest pain radiating to left arm, uncomfortable pressure on chest with shortness of breath, fatigue, sweating, headache with altered consciousness, numbness, weakness of one or both side of body. Medical examination was followed by neurological and chest examination. Patients giving history of chest discomfort was advised electrocardiogram (ECG) to look for the changes in ECG. If diabetic patients gave any of the above history, they were excluded from the study.

Minor comments

Comment 1. In page 9 line 14, please check the final concentrations of primers, 0.5 mM.

Answer to Comment 1. It is a typographical error. Corrections have been made on Page 8, Line no: 18

Comment 2. In page 11 line 6, please define 'HC'.

Answer to Comment 2. It is a typographical error. It should be NG (Normoglycemic) instead of HC. Correction have been made on Page 10, Line no:9

Comment 3. In page 11 lines 24-26, which -94 ATTG insertion/deletion allele links to these autoimmune diseases?

Answer to Comment 3. Deletion allele of -94 ATTG insertion/deletion is associated with decreased risk of developing various inflammatory and autoimmune diseases.

Comment 4. In page 12 line 8, please define 'viz.'?

Answer to Comment 4. Viz. shortform for the word videlicet and is used as a synonym for "namely". However for the convenience of readers the word "viz." has been replaced with "namely" on Page 11, Line no: 25.

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