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ROLE OF NF-kB POLYMORPHISM AND GLOBAL DNA METHYLATION IN SCREENING GESTATIONAL DIABETES MELLITUS

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Abstract

INRODUCTION:Gestational diabetes mellitus has emerged as a global public health problem with an exceptionally high estimated prevalence.The addition of biomarkers to predictive models of GDM may improve the ability to identify women at risk of GDM prior to its development.SETTING:SCB Medical College, Cuttack, Odisha, India.OBJECTIVE:To study the role of NF-kB and DNA-methylation as biomarkers in gestational diabetes mellitus.MATERIAL AND METHOD:Eligible participants were >18 years old with singleton pregnancy, plan to carry the pregnancy to term, and planned to deliver at our institution.Data was collected using in-person interviews and antenatal checkups. Information was collected on sociodemographic characteristicsThe study was a prospective observational study and enrolled 100 pregnant women after screening for the exclusion criterias.DNA extraction followed by Restriction fragment Length Polymorphism (RFLP) and DNA-methylation quantification was done in Molecular research unit(MRU). RESULTS AND CONCLUSION: We found the prevalence of GDM to be 34%.Three types of polymorphism were found. The most common type was ins/ins type (53%) followed by del/del type (32%) and ins/del type in only 15 %..This study provides the evidence that NF-kB polymorphism and global DNA-methylation has no predictive value for screening GDM.

Keywords: Gestational Diabetes Mellitus, NF-kB Polymorphism, DNA Methylation.

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Gestational diabetes mellitus has emerged as a global public health problem with an exceptionally high estimated prevalence in India. (27.5%)1. Nearly 4 million pregnancies are complicated annually in India alone representing a large subset of population at high risk for adverse perinatal morbidity and mortality if left inappropriately managed2. Considering the potential adverse outcomes in both mother and offspring and wide range of GDM prevalence in the country, its early identification assumed national significance.Early detection of GDM may lead to improved management, possibly preventing pregnancy complications. Thus, the identification of sensitive and specific biomarkers, which may offer potential for risk

prediction and intervention strategies, became a major focus in GDM research3.

In recent years, molecular biomarkers have received considerable interest as screening tools for GDM. The addition of biomarkers to predictive models of GDM may improve the ability to identify women at risk of GDM prior to its development. An increasing number of publications have proposed the abnormal epigenetic modifications as biomarkers in the pathophysiology of metabolic diseases, including GDM4. It has been hypothesized that epigenetic mechanisms could fill the knowledge gap between environmental factors (i.e., diet, pollution, stress, smoke and others) and heritable genetic susceptibility5.

A growing body of evidence supports the use of Single nucleotide polymorphisms (SNPs), DNA methylation, and miRNAs as biomarkers3.

The purpose of this study is to assess the role of single-nucleotide polymorphisms (eg.NF-kB) and epigenetics (e.g global DNA-methylation) as biomarkers for GDM. Understanding of the molecular pathophysiology will enhance the possibility of effective screening, early intervention, and even prevention.

Screening and Diagnosis of GDM:

Different methods for screening and diagnosis of GDM have been proposed by international societies; there is controversy regarding the diagnosis of GDM by either the One Step or the Two Step approach6.The methods are as followed:

Society	Test	Glucose load(gm)	FBS Mg/dl	1Hr PPBS Mg/dl	2Hr PPBS Mg/dl	3Hr PPBS Mg/dl	No. of abnormal values required for diagnosis of GDM
DIPSI 2005	1 step	75	-	-	140	-	1
WHO 2013	1 step	75	92	180	153	-	≥1
FIGO2013	1 step	75	92	180	153	-	≥1
IADPSPG2015	1 step	75	92	180	153	-	≥1
NICE/RCOG2015	1 step	75	101	-	140	-	≥1
ACOG/C&C2017	2 steps	100	95	180	155	140	≥2
ACOG/NDDG	2steps	100	105	190	165	145	≥2
ADA 2017	1 step	75	95	180	155	-	≥2
ADA 2017	2steps	100	95	180	155	140	≥2

Govt of India endorses Diabetes in Pregnancy Study Group India (DIPSI) criteria and employed single-step procedure in guidelines for GDM diagnosis7. In accordance to DIPSI criteria, 2-h 75-g OGTT was employed to all pregnant women irrespective of their last meal timings. Although the criteria for GDM screening and diagnosis were established, uncertainty still existed on its execution methods.

The pathophysiologic changes occur early in pregnancies that are later complicated by GDM and/or other related disorders8. These pathophysiologic changes include systemic changes (including chronic systemic inflammation and dyslipidemia) and are reflected in peripheral blood. Further understanding of the cellular mechanisms that occur during pregnancy and their dysfunction in GDM may direct meaningful searches for biomarkers that have prospective clinical utility

Nuclear factor-κB (NF-κB) was initially identified to be located on chromosome 4q23-q24 in 1986 as a transcription factor which binds to a 10 bp DNA element in kappa immunoglobulin light-chain enhancer in B cells9. It is a ubiquitous dimeric, redox-sensitive transcription factor that regulates the expression of more than 160 target genes which are mainly involved in immune and inflammatory response and apoptosis. Its main function is to recruit circulating monocytes into the subendothelial cell layer of the blood vessel wall. It has been reported to be involved in the

pathogenesis of atherosclerosis, cardiovascular disease, obesity and insulin resistance10.

Prior to stimulation, NF-κB dimers reside primarily in the cytoplasm as an inactive complex with nuclear factor-κ B inhibitor (IκB). In response to extracellular stimuli, IκB is phosphorylated. This phosphorylation allows the NF- κB dimer to dissociate and translocate to the nucleus where it can bind DNA promoter sequences of pro-inflammatory genes and upregulates the target genes that encode inflammatory mediators such as TNF-α, IL-1β, and IL-611 . Five members in the NF-κB(transcription factor) family have been identified, and they are NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), RelB and c-Rel. Before the NFkB complex is translocated into the nucleus, NFkB1 and NFkB2 are cleaved to the active p50 and p52 subunits, respectively. The target gene specificity of NF-κB is determined by the subunit type. NF-κB is involved in both pro-inflammatory and anti-inflammatory processes by encoding both the p105 and p50 subunits by alternative splicing12. As a part of the p50/p65 NF-κB transcription factor complex, it is pro-inflammatory, by stimulating the transcription of proinflammatory cytokines such as TNF-α and IL-1β13 . In contrast, p50 has antiinflammatory properties in the p50 homodimer (p50/p50), which represses transcription of pro-inflammatory cytokines and stimulates transcription of the anti-inflammatory cytokine IL-10.14

Inflammatory pathways linking inflammation to insulin resistance. Activation of JNK and NF-κB pathways causes serine kinase phosphorylation of IRS-1 or IRS-2, which may block insulin signaling and finally lead to the occurrence of IR. In addition, JNK and NF-κB pathways are involved in the production of proinflammatory cytokines which may in turn become activation stimuli of the pathways15.

Several polymorphisms including -94ins/del ATTG in the NFKB1 promoter have been identified. Current knowledge reveals that functional NFKB1 promoter SNP consisting of a insertion/deletion (−94 ins/del ATTG) (rs28362491) as regulatory effect on the NFKB1 gene16.

Recently, two different in vitro studies17,18 observed that an insertion of an ATTG sequence at position -94 of the promoter region of the NFKB1 gene increases transcriptional activity compared to the allele with the deletion. In humans, the production of the p105 and p50 proteins has been shown to be greater among individuals with the ins/ins genotype. It has been shown that the deletion of the ATTG repeat in the promoter region of the NFKB1 gene results in lower promoter transcriptional activity and causes a decrease in p50 subunit levels18. Therefore, it can affect both the availability of the proinflammatory p50/p65 NF-κB heterodimer and anti-inflammatory p50/p50 NFκB homodimer. A common ins/del (−94 ins/del ATTG rs28362491) polymorphism of NFKB1 gene promoter exerts functional effects on the transcription of the NFKB1. Among various NF-κB inducers, the toll-like receptors (TLRs) seem to be key initiators of innate immunity, recognizing certain pathogen-associated lipopeptides, peptidoglycans, and other lipid moieties and then triggering a cascade of cellular signals, culminating in the activation of NF-κB .

Since NF-κB is responsible for the regulation of many other genes in disease progression, variants in the genes coding for the NF-κB and IκB proteins of could be potentially involved in disease development. Since the 4-bp ins/del polymorphism produces a relatively large sequence change and its location is proximal to binding sites that are important to promoter regulation, the ATTG deletion (D) allele displays significantly reduced promoter activity and it is also involved in lower levels of p50 protein. Thus, this polymorphism seems to be relative to several inflammatory diseases16,17. Polymorphism in the NFKB1 promoter region at position -94 ins/del AGGT has been correlated with many long standing inflammatory diseases like autoimmune diseases such as rheumatoid arthritis, asthma, AIDS, cancers and various diabetic complications19 .Increased expression of NF-kB pathway in placentae of GDM patients, increased TLR expression in peripheral blood

mononuclear cells of GDM patients that activates NF-kB could aid in the early detection of GDM, thus facilitating intervention strategies to better manage GDM and improve health outcomes. In this study, we focus on polymorphisms of the NFKB (encoding for NF- κB) and NFKBI (encoding for IκB) in relation to development of GDM.

One suggested mechanism by which SNPs (NF-kB polymorphism) change gene expression is through epigenetics (DNA-methylation) via the introduction or removal of CpG sites. Histone acetylation of NF-κB target genes is generally associated with increased binding of the transcription factor to its response elements and active transcription.

OBJECTIVES

1. to study the role of NF-kB and DNA-methylation as biomarkers in gestational diabetes mellitus.

2.To study the association of NF-kB polymorphism and gestational diabetes mellitus.

3. To study the association of global DNA-methylation and gestational diabetes mellitus.

MATERIALS AND METHODS:

This study has been approved by Institutional Ethics Committee, S.C.B. Medical College, with Ethics Committee Regd. No. ECR/84/Inst/OR/2013/RR-20 .Written, informed consent was obtained from all partaking mothers prior to data collection.

Peripheral blood samples was obtained from Participant pregnant women who initiated prenatal care between 13- 24 weeks of gestation from January-2019 to December-2020.

Eligible participants were >18 years old with singleton pregnancy, plan to carry the pregnancy to term, and planned to deliver at our institution.Data was collected using in-person interviews and antenatal checkups. Information was collected on sociodemographic characteristics (eg, age, race/ethnicity, and education) and physical activity were taken by questionnaire. Height was measured and information on the pre-pregnancy weight was taken from the MCP card. We used last menstrual period-based dating (confirmed by early pregnancy ultrasound-based dating) to estimate date of conception and gestational age. At the end of pregnancy, medical records were abstracted to obtain information on course and outcomes of the pregnancy.

Study participants underwent a screening test, a 75-g 2-hour oral glucose challenge test irrespective of the last meal, at 1st visit followed by a similar repeat test at 24-32 weeks of gestation on subsequent visit. Any pre-pregnancy blood sugar level if done was recorded to rule out prior Type-2DM. GDM was diagnosed according to DIPSI criteria.

DNA extraction followed by Restriction fragment Length Polymorphism (RFLP) and DNA-methylation quantification was done in Molecular research unit(MRU),Department of Biochemistry, SCB Medical College,Cuttack.

Exclusion Criteria:

1. patients less than 18 years of age

2. patients at GA <13 weeks and >24 weeks.

3. diagnosed cases of GDM

4. multiple gestations

5. Subjects having T-2 DM and macrovascular complication of diabetes like

CAD, stroke

6. Presence of autoimmune disorders,

7. Kidney disease (hypertensive nephropathy, chronic glomerular nephritis,

tubule interstitial disease, obstructive uropathy, CCF, malignancy, liver

disorders).

8. Infections

9. diagnosed cancer

METHODS:

The study was a prospective observational study and enrolled 100 pregnant women after screening for the exclusion criterias.

Blood glucose estimation:

Blood was collected and was kept in clot activator vials for serum biochemical analysis, in EDTA treated vials for molecular techniques and in oxo-fluoride vials for plasma glucose. Plasma glucose levels were estimated by GOD-POD (Glucose oxidase peroxidase) method adapted to autoanalyzer (Toshiba 120FR, JAPAN).

DNA extraction:

Blood samples of 2 ml were collected in blood collection tubes treated with EDTA. Genomic DNA was extracted from peripheral blood leukocytes using a DNA isolation kit according to manufacturer's protocol (Genomic DNA isolation Kit, Genxbio) and stored at 40C.

Polymorphism genotyping:

To determine polymorphism, regions of 285 bp in NF-κB1 were amplified using the following primer sets (Forward 5'- TGGGCA CAAGTCGTTTATGA-3' and Reverse 5'CTGGAGCCGGTAGGGAAG-3')20. PCR was performed by amplifying 20 ng of DNA in a 50 µl reaction, 19 PCR buffer, 2 mM MgCl2 (NF-κB1), 0.5 mM dNTPs, 0.5 µM each primer, and 0.5 U Taq DNA polymerase. The amplification conditions were 1 min at 95˚C, 30 s at 95˚C, 30 s annealing at 60˚C, 1 min at 72˚C, and finally 5 min at 72˚C, for 35 cycles. (VERITI 96 WELL THERMAL CYCLER, THERMOFISHER SCIENTIFIC, APPLIED BIOSYSTEMS, USA).

For detection of the −94 ins/del ATTG polymorphism in NF-κB1, a PCR product

of 281/285 bp was digested with restriction enzyme PfIMI (10 U/µl, Fermentas), which has a recognition site in this region. Five microliters of PCR product was

digested by PfIMI in a 25 µl reaction (2.5 µl 109 buffer, 0.3 µl PfIMI) at 37˚C

overnight. There are two ATTG repeats at its promoter region, one allele of

which has an ATTG insertion (ins). Therefore, one unique recognition site takes

the form 5'…CCANNNN NTGG…3' at the ins allele. Consequently, the ins allele

was cleaved into two fragments of 45 bp and 240 bp after restriction digestion.

However, there was no cleavage at the deletion allele (del) that has only one ATTG at its promoter. The bands of digested products were visualized in 2% agarose gel electrophoresis stained with ethidium bromide.

Genome-wide DNA methylation profiling:

The extracted DNA is bound to strip wells that are specifically treated to have a high DNA affinity. The methylated fraction of DNA is detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of methylated DNA is proportional to the OD intensity measured. The assay was done by ABNOVA methylated DNA quantification kit(calorimetric) KA1510 version 05. After preparing the DNA sample,it was gently mixed with prepared reagents in strip wells. Strip plates were covered with plate seal or Parafilm M and incubate at 37°C for 90 min. The binding

reaction solution was removed from each well by washing each well with sequential amount of the Diluted ME1 1X Wash Buffer followed by incubation each time. Color change in the sample wells and control wells was monitored after adding reagents and incubation. The ME8 solution will turn blue in the presence of sufficient methylated DNA and yellow in ME9 solution. The absorbance should be read on a microplate reader at 450 nm within 2 to 15 min.

DATA ANALYSIS:

Calculation of Results :

Relative Quantification: To determine the relative methylation status of two different DNA samples, simple calculation of percentage of 5-mC in total DNA can be carried out using the following formula:

5-mC% = (sample OD – ME3 OD)/ S X 100%

(ME4 OD – ME3 OD)x 2/ P

450 nm OD: **ME3**= 0.9040

ME4= 3.3990

S= amount input sample DNA (ng) , P= amount input ME4 (ng) = 10 ng

Statistical Analysis:

Participant characteristics were described using mean (standard deviation) and

number (percentage, %) for continuous and categorical variables, respectively.

The data were analyzed using SPSS version 21 and correlation was done using

spearman test. The p-values were calculated from chi-square charts. Confidence

interval were calculated. Confidence interval of 95% and the level at $P < 0.05$

were considered significant.

OBSERVATIONS AND RESULTS:

In the current study,100 pregnant females were studied, out of which 34 cases developed GDM on follow up. The prevalence of GDM in this study was 34%.

The mean blood glucose level in GDM patients in the study was 179.00 mg/dl with SD=30.93 and in non-GDM was 101.52mg/dl with SD=17.34 . The p-value was <0.05(0.0001). (Table-1)

Table-1: *Blood sugar levels:*

Blood sugar	Mean	SD	P value
GDM present	179.00	30.93	0.0001
GDM absent	101.52	17.34	
Total	127.86	43.32	

According to BMI calculated at the first antenatal visit, the mean BMI was 25.9kg/m2. Most of the women(48) were of normal BMI,22 were overweight and 30 were obese. Obese women had significantly higher risk(53.33%) for GDM followed by overweight(32.35%)compared to normal weight women (20.58%). P-value for BMI ≥ 25 was 0.0001 and so was associated with development of GDM.(Table-2)

Table-2: *Association of GDM and BMI*

BMI	GDM present	GDM absent	Total	P value
<25	7	41	48	0.0001
≥25	27	25	52	
Total	34	66	100	

NF-KB POLYMORPHISM IN THE STUDY GROUP:

NF-kB polymorphism and GDM: Genotype status was obtained for the 100 participants. The following types of polymorphism were seen: Three types of polymorphism were found. The most common type was ins/ins type (53%) followed by del/del type (32%) and ins/del type in only 15 %.(Table-3)

Table-3: *NF-kB Polymorphism and GDM*

NF-kB	Number	Percentage
INS/INS	53	53.0
INS/DEL	15	15.0
DEL/DEL	32	32.0
Total	100	100.0

(A) ins/ins type (Table-4)

Table -4: *ins/ins type polymorphism:*

	GDM present	GDM absent	Total	P value	OR(CI)
ins/ins(+)	21	32	53	0.208	1.716 (0.738-3.990)
ins/ins(-)	13	34	47		
Total	34	66	100		

The positive predictive value of NF-kB ins/ins type polymorphism is 39.6% whereas negative predictive value is 72.3% resulting in a sensitivity and specificity of 67% and 51.5% respectively with p-value of >0.05(0.208).

(B) ins/del type(Table-5)

Table-5:*ins/del type polymorphism:*

	GDM present	GDM absent	Total	P value	OR(CI)
ins/del(+)	3	12	15	0.024	0.435 (0.114-1.663)
ins/del(-)	31	54	85		
Total	34	66	100		

The positive predictive value for ins/del type is 20% whereas negative predictive value is 63.5% with sensitivity and specificity of 8.8% and 81.8% respectively with p-value of >0.05(0.214).

(C) del/del type(Table-6)

Table -6: *del/del type polymorphism*

	GDM present	GDM absent	Total	P value	OR(CI)
del/del(+)	10	22	32	0.690	0.833 (0.340-2.045)
del/del(-)	24	44	68		
Total	34	66	100		

For del/del type of polymorphism,the positive predictive value is 31.2% and negative predictive value is 64.7% with sensitivity and specificity being 29.4% and 66.7% respectively an p-value >0.05(0.690).

Association of DNA-methylation with GDM:

Mann Whitnwy U test was used to compare both the groups and found the mean DNA-methylation to be 0.52 with SD 0.32 and a p-value of 0.834.(Table-7)

Table -7: *Association of DNA Methylation with GDM:*

DNA Methylation	Mean	SD	P value
GDM Present	0.525	0.341	0.834
GDM absent	0.511	0.281	
Total	0.516	0.302	

DISCUSSION

Although the underlying mechanisms remain to be studied, growing evidence suggests that systemic and local production/action of inflammatory molecules may be particularly critical in causing IR in GDM. Several inflammatory pathways participated in local IR. The TLR4 pathway is a possible candidate which can induce

a potential pro-inflammatory reaction. Patni S et al (2009)21 reported positive association of NF-kB with placental expression of TLRs in normal term placenta, a key transcription factor in TLR-mediated inflammation. Yun Jet al(2011) has shown to induce NF-κB activation in monocytes with associated increases in NF-κB target genes such as the pro-inflammatory TNF-α and IL-6 in diabetic individuals. Kuzmicki et al(2013)22 found positive correlation of TLR4 mRNA expression in PBMCs in normoglycemic women who subsequently develop GDM, however it becomes insignificant after 4 weeks when normal healthy individuals also express the same.

Xie, B et al,(2014) observed a positive correlation between TLR4 mRNA expression and serum TNF-α levels in blood monocytes in a sample size of 60 females which included 32 GDM patients. Mrizak I et al(2014)23 and Feng H et al(2016)24showed positive correlation between enhanced expression of TLR4/MyD88/NF-κB with insulin resistance in placentae of gestational diabetes mellitus. Dasu et al(2018)21has documented the role of TLR4 that activates NFkB pathway. Our study to the

best of our knowledge is a novel study that has studied for the first time the involvement of NF-kB gene polymorphism in peripheral blood mononuclear cells in a relatively larger study group that included GDM cases. However the pathway involving NF-kB needs further extensive study.

The study detected three types of polymorphism, the insertion/insertion (ins/ins), insertion/deletion (ins/del) and deletion/deletion (del/del). The ins/ins type(53%) was found to be the most common in our study group followed by the del/del type(32%) and then the ins/del type(12%). All the three types of polymorphism were detected both in the GDM and normoglycemic patients. We found no type specific to GDM.

The p-value for ins/ins type polymorphism for GDM and non-GDM group was >0.05(0.208) with odds ratio(CI) of 1.716(0.738-3.990) shows no positive association with GDM. The sensitivity of ins/ins being highest (67%) when compared with that of ins/del (8.8%) and del/del (29.4%);indicating more true positive rates. Similarly, the specificity was calculated to be the lowest (51.5%)in ins/ins type, 81.8% in ins/del and 66.7% in del/del type. This indicates ins/del has highest true negative rate. The positive predictive value for ins/ins is 39.6%, ins/del was 20% and del/del is 31.2 %;and the negative predictive value for ins/ins, ins/del and del/del was 72.3%,63.5% and 64.7%.The respective p-values/OR(CI) were 0.214/0.435(0.114-1.663) for ins/del and 0.690/0.833(0.340-2.045) for del/del.

Thus no polymorphism is found to be associated with presence of GDM and hence cannot be used as a screening tool. However an extended study on larger group and better understanding of the molecular mechanism can give insight. Bouchard L et al(2012,2013)25 studied the positive correlation of genomic methylation in placental tissue adiponectin gene in GDM and MEST gene hypomethylation in cord blood and placental tissue. Hajj El et al(2013)26 genomic study of leptin gene in placental tissues and maternal and cord blood showed a positive correlation with GDM. . On the other hand Matsha et al.(2016),27who showed no difference in global DNA methylation between GDM women and normoglycemic one in the same population. The p-value for DNA-methylation in our study was found to be 0.834 thus, indicating no significant correlation. Most of these studies were conducted on placental tissue and used different methods to quantify global DNA methylation, possibly accounting for the discrepancies observed. Biological source affects global DNA methylation, thus our failure to observe an association between GDM and global DNA- methylation could be due to the use of peripheral blood cells rather than placenta.Although quantification of global DNA methylation is a robust method to assess overall genomic DNA methylation, and has potential as a biomarker to facilitate risk stratification and intervention, it may not offer the resolution required to detect subtle methylation differences in women with or without GDM.

DNa-methylation and NFkB polymorphism requires further research and study to be incorporated as a screening tool for gestational diabetes mellitus if at all.

CONCLUSION

We found the prevalence of GDM to be 34%. Blood glucose estimation by DIPSI method proved to be a better screening tool when compared to the NFkB polymorphism and DNA-methylation . NFkB polymorphism and DNA-methylation were not found to have any positive predictive value as a screening tool to diagnose gestational diabetes mellitus. Although there are gaps in our knowledge of the mechanisms involved, in this study, we described a comprehensive view of the relationship between NF-kB polymorphism and DNA-methylation with development of GDM. This study provides the evidence that NF-kB polymorphism and global DNA-methylation has no predictive value for screening GDM. Our findings may provide some insight for more targeted approach toward developing simple biomarkers for predicting GDM, thus facilitating intervention strategies in the early

antenatal period to improve the health of the mother and baby, both during pregnancy and in the long-term.

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