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FETAL MMP2 POLYMORPHISM AND THE RISK OF FETAL GROWTH RESTRICTION

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ABSTRACT

In pregnancy poor embryo implantation and poor placentation can cause inadequate exchange between fetus and mother,that cause fetal growth restriction.Matrix metalloproteinases are proteases that causes degradation of extracellular matrix and involved in embryo implantation.Aim of this study is to evaluate association between Maternal sociodemographic factor fetal clinical parameter ,genotypic mutation and fetal growth restriction.Total 85 patients chosen ,65 control who gave birth to AGA newborn and 20 mother who gave birth to FGR newborn. Different maternal sociodemographic and newborn clinical parameter assessed and by fetal cord blood during birth genotypic study carried out, DNA isolated and analysed by PCR followed by restriction enzyme digestion.The risk of FGR increase in CT heterozygous as compare to CC genotype (odds ratio-1.14),so we concluded that fetal MMP2 -1306 single nucleotide polymorphism is associated with at risk for developing fetal growth restriction.

KEYWORDS :

INTRODUCTION

The normal neonate is one whose birth weight is between the 10th to 90th percentile at given gestational age, gender and race and having no feature of malnutrition and growth retardation in utero.

Fetal Growth restriction previously called "Intrauterine growth restriction". Fetal Growth Restriction is defined as a pathological condition in which a fetus has not achieved his genetic growth potential regardless of fetal size. Small for gestation age is often used as being a proxy for IUGR. Smallness is often defined as an estimated fetal weight (EFW) or abdominal circumference (AC) less than 10th percentile and severe SGA as EFW or AC less than 3rd percentile. FGR is sometimes defined as SGA with abnormal Doppler indices.

Normal pregnancy is associated with several utero-placental and Normal pregnancy is associated with several utero-placental and hemodynamic changes in order to meet the growing and metabolic demands of the developing foetus. The pregnant uterus undergoes hypertrophy and distension in order to provide sufficient space for the growing foetus. Placental remodelling and cyto-trophoblast invasion of spiral arteries maintain adequate blood supply to the developing foetus.[1,2] Also, the increases in maternal blood volume and cardiac output are balanced by systemic vasodilation and decreased vascular resistance, which leads to minimal change in blood pressure.[3,4] These uterine and vascular changes involve marked utero-placental and vascular remodelling and redistribution of blood flow in different maternal organs.[5,6]

Mmp 2 And Pregnancy

Matrix metalloproteinases (MMPs) are zinc-dependent proteases that play a role in tissue remodelling. MMPs include collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs with different tissue expression, distribution and substrate specificity. MMPs degrade different proteins in the extracellular matrix (ECM) like collagen and elastin. MMPs are produced as pro-MMPs which are cleaved by other MMPs or proteases into active MMPs. MMPs play an important role in endometrial tissue remodelling and may be involved in the uterine and vascular tissue remodelling during normal pregnancy.

Both MMP2 (gelatinase A/72 kDa type IV collagenase) and MMP9 (gelatinase B/92 kDa type IV collagenase) genes are expressed in first trimester trophoblast cells with MMP2 abundance decreasing and MMP9 abundance increasing over time[7,8]. In the precise time-frame corresponding to embryo implantation, both metalloproteinases enable the penetration of cytotrophoblasts in maternal endometrium and the first third portion of myometrium by degrading maternal ECM[9].

2.MATERIAL AND METHODS

The present study was carried out in Department of Obstetrics and Gynecology, Institute of Medical Sciences, Banaras Hindu University, and in Department of Molecular and Human Genetics, Banaras Hindu University.from july 2017 to December 2018.

The study group consists of 85 patients (20 FGR cases and 65 controls), attending antenatal clinic or labor room in Obstetrics and Gynecology ward SSH, BHU. Informed consent was obtained and approval of the university's ethical committee for research on Human material was obtained.

Patients

Cases: Patients attending antenatal clinic or labor room of Department of Obstetrics and Gynecology , SSH, IMS, BHU with age group 18-45 years delivering a baby at term whose birth weight at term less than 10th percentile or clinical features of growth restriction or abnormal Doppler indices.

The diagnostic procedures which will be used are mentioned below…Detailed history & clinical examination, Urinary pregnancy test, Serial Ultrasonography with Color Doppler.

Inclusion criteria were patients of singleton pregnancy with age group 18- 45 years delivering a baby with birth weight more less than 10th percentile, at term gestation or with feature of growth restriction.

Exclusion Criteria were females with complications such as multiple pregnancy,abnormal fetal karyotype,endocrine abnormalities, history of autoimmune disease, major fetal malformation ,maternal diseases such as diabetes mellitus, previous or present high blood pressure, kidney disease etc and TORCH infection. According to these inclusion/ exclusion criteria, we obtained 20 FGR cases. In cases and control gestational age was based exclusively on first trimester ultrasound, between 8th to 10thweek of gestation Informed consent was obtained and approval of the university's ethical committee for research on Human material was obtained. Before inclusion in this study, objective evidence of past and present pregnancy was asked which included original lab reports of positive HCG test, ultrasound

Doppler report of pregnancy, all relevant questions asked from patient or family members.

Anthropometry;

The data was collected between the time of birth to the day of discharge.

I. **Birth Weight :** Birth weight of the new born was recorded in grams by electronic balance with a difference of ±10 gm within 12 hours of birth.
II. **Crown-heel Length:** crown heel length was recorded to the nearest of 0.1 cm with an infantometer with the baby being supine, knees fully extended and soles of feet held firmly against the foot board and head touching fixed board.
III. **Head Circumference:** The head circumference was measured by passing a non-elastic tape over the occipital protuberance on the back and supraorbital ridges in front.
IV. **Abdominal Circumference:** It was measured at the level of umbilicus with same non elastic tape.
V. **Mid Arm Circumference:** It was measured by marking with a pen the olecranon process and acromium. Mark the mid-point between these two marks, wrap a tape around the arm at the midpoint.

Molecular And Genetic Analysis;

Collection Of Samples: -

Collection Of Blood Samples: - Cord blood (3 ml) collected in plain vial and EDTA vial from fetuses of cases and control.

Techniques To Be Employed-.

DNA Isolation From Blood Sample- 1 ml blood was taken in 15 ml falcon tube. 1 ml 0.85 % NaCl was added in it and mixed well with invert mixing. This is done to wash the sample as the concentration of salt solution is isotonic to the blood. Tubes were centrifuged at 7000 RPM for 7 minutes at 25°C. Supernatant was discarded and pellet was broken. 1 ml of solution A was added and mixed well with pellet by gentle invert mixing for few minutes. This solution known as hemolysis buffer contained 0.32 M sucrose hypotonic to the RBCs to cause their lysis. Tubes were centrifuged at 7000 RPM for 7 minutes at 25°C. Supernatant was discarded and pellet was re-suspended in 400 ul of solution B (nucleolysis solution) by gentle invert mixing. Solution C (100 μl) was then added and mixed with gentle inversion. 400μl of ice chilled chloroform was then added and mixed with gentle inversion so that two phases are mixed properly with a milky appearance. Tubes were then subjected to centrifugation at 7000 RPM for 7 minutes at 25°C. Upon centrifugation, three layers were produced: an upper aqueous phase, a lower organic layer, and a compact band of denatured protein at the interface between the aqueous and organic phases. The aqueous layer was carefully taken out in separate eppendorf tube and approximately equal amount of isopropanol is added to precipitate out the DNA. Thread like DNA was then carefully pooled into the eppendorf tube by a cut tip pipette.It was then washed with 70% alcohol and then followed by centrifugation at 10000 RPM at 25°C for 10 minutes. Alcohol was discarded and it was left to dry for few minutes. Finally, DNA was dissolved in MQ according to the amount of DNA precipitated. DNA samples were run on 0.8% agarose gel to check the quality of DNA isolated.

Genotyping the SNP of MMP2 C-1306Twas analyzed using Polymerase Chain Reaction & Restriction Fragment Length Polymorphism (PCR-RFLP).

PCR And RFLP Conditions Used Are:

For MMP2 C-1306T :

Step 1 : Denaturation step for 5 min at 94^{0}C
Step 2 : Denaturation for 30 seconds at 94^{0}C
Step 3 : Annealing for 37 seconds at 60°C
Step 4 : Extension for 33 seconds at 72^{0}C
Step 5 : Go to step 2-4 for 35 times
Step 6 : Final extension for 10 min. at 72^{0}C
Step 7 : 4^{0}C for infinite in a thermocycler.

The PCR product of 193 bp of was digested with Bfa1 restriction enzyme at 37°C in water bath for 12 hours. Components of restriction digestion reaction mixture is given in Table below. The digested products were separated on a 4% agarose gel.

Details Of Primers Used In PCR Is Given Below:

Primer sequence , MMP2 C-1306T	PCR product
Forward primer 5'- CTT CCT AGG CTG GTC CTT ACT GA- 3' Reverse primer 5'- CTG AGA CCT GAA GAG CTA AAG AGC T- 3'	193bp

Statistical Analysis:

Clinical parameters .phenotype, allele and genotype distribution among groups were evaluated using chi-square test. A two-tailed p-value of ≤0.05 was considered as statistically significant. .All stastistical analysis were performed using SPSS 16 software.

3. RESULTS

3.1 Patients-

20 FGR cases with a newborn birth weight under the 10th percentile and 65 AGA controls with a newborn birth weight between the 10th and the 90th percentile were enrolled in study.

Table 1 : Distribution Of Mean Of Different Sociodemographic Factors-

Factors	Group	Mean	Std. Deviation	p value
Age(years)	Fetal growth restriction	23.85	4.771	0.327
	Control	24.89	3.236	
Gravidity	Fetal growth restriction	1.70	1.031	0.648
	Control	1.58	.864	
Gestational age(weeks)	Fetal growth restriction	38.10	1.071	0.118
	Control	38.58	1.261	
Pre pregnancy weight(kg)	Fetal growth restriction	54.45	10.836	0.110
	Control	57.42	5.068	
Weight gain during pregnancy(kg)	Fetal growth restriction	6.25	1.650	0.01
	Control	8.82	1.117	
Symphysial fundal height(cm)	Fetal growth restriction	31.47	1.826	0.01
	Control	37.36	.866	

p value <0.05 is considered as significant.

On comparison of mean of different parameters in FGR group ,mean weight gain during pregnancy (kg)in cases was 6.25±1.650,in control it was 8.82±1.117,which was significant, there was also a significant difference in in symphysial fundal height (cm) in case (31.47±1.825) and control (37.36±0.866).

Table 2: Distribution Of Different Maternal And Sociodemographic Factors In Comparison Group

Study parameter		Fetal growth restriction		Control		p value
		No.	%	No.	%	
Place pf Residence	Rural	4	20	20	30.8	0.262
	Urban	16	80	45	69.2	
Educational status of	Illiterate	8	40	5	7.7	0.001
	Upto10th	9	45	28	43.1	

mother	> 10standard	3	15	32	49.2	
Socioeconomic status	Lower middle	14	70	25	38.5	0.013
	Upper middle	6	30	40	61.5	
Iron Folic Acid intake	No	5	25	16	24.6	0.593
	Yes	12	75	49	75.4	
Previous similar history	No	14	70	59	90.8	0.030
	Yes	6	30	6	9.2	
Maternal height	> 145 cm	8	40	38	58.5	0.117
	< 145 cm	12	60	27	41.5	
Body mass Index	> 25	17	85	58	89.2	0.432
	< 25	3	15	7	10.8	
ANC visit	No or≤ 2	11	55	26	40	0.423
	≥ 3	9	45	39	60	

On comparison of different sociodemographic factors FGR was more common in illiterate (40%) than studied more than 10th standard (15%),in FGR group 30% and in control group only 9.2% patients having previous similar history .

Table 3- Distribution Of Weight Gain During Pregnancy In Groups-

Weight gain during pregnancy (kilograms)	Fetal growth restriction		Control	
	No.	%	No.	%
0-5	9	45	0	0
5.1-8	8	40	28	43.1
8.1-10	3	15	32	49.2
More than 10.1	0	0	5	7.7
Total	20	100	65	100

(p value=0.0001), p value <0.05 is considered as significant.

Table 4: Comparison Of Mean Of APGAR Score After 1 Minutes And 5 Minutes

APGAR Score	Groups	Mean	Std. Deviation	p value
APGAR after 1 minutes	Fetal growth restriction	5.95	1.317	0.01
	Control	7.83	.417	
APGAR after 5 minutes	Fetal growth restriction	7.65	1.268	0.00
	Control	8.92	0.269	

Table 5: Comparison Of Mean Of Different Anthropometric Parameters Of Newborn

Parameters	Groups	Mean	Std. Deviation	p value
Head circumference(cm)	Fetal growth restriction	31.79	.720	0.00
	Control	34.46	.532	
Abdominal circumference(cm)	Fetal growth restriction	29.650	.7251	0.00
	Control	33.343	.5034	
Crown heel length(cm)	Fetal growth restriction	45.000	.7760	0.00
	Control	49.683	.6400	
Mid arm circumference(cm)	Fetal growth restriction	8.830	.3389	0.00
	Control	10.434	.4836	

On comparison of APGAR score (Table 4) in 1 and 5 minutes after birth significant difference was found,and different newborn parameters like crown heel length,head.abdominal and mid arm circumference there was also significant difference found.

3.2 MMP2 Polymorphism

Table 6: -Distribution of genotypes and allele frequencies of MMP2 C>T polymorphism in study population.

Genotype	Case (IUGR) (n=20)	Control (Healthy individuals) (n=65)	OR	95% CI	p value (Yates corrected)
CC	12 (0.60)	41 (0.63)	1	-	-
CT	8 (0.40)	24 (0.37)	1.14	0.40-3.17	1
TT	0 (0)	0(0)			0
Allele frequency					
C	32 (0.80)	106 (0.82)	1	-	-
T	8 (0.20)	24 (0.18)	1.10	0.45-2.69	1

Note: OR=odds Ratio; CI=95% confidence interval.

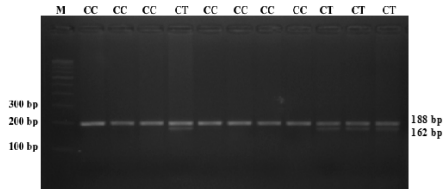
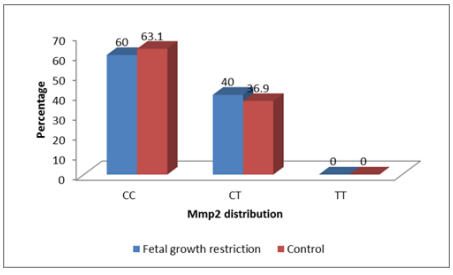


Figure 15a : MMP2 (C>T) polymorphism was detected by PCR-RFLP. The 193 bp PCR product was digested with Bfa1 restriction enzyme. The T allele gets cut and yields 162 bp and 26 bp and 5 bp product and C allele cuts gets cut and yields 188 bp& 5 bp product. Lane 1 shows 100 bp marker, Lane 5,10,11,12: CT heterozygous, Lane 2,3,4,6,7,8,9: CC homozygous (wild type).



Bar graph showing the distribution of distribution of MMP2 C>T polymorphism in IUGR and control patients.

Genotypes, CT of MMP2 C>T polymorphism, shows a slight increase in risk towards the risk of IUGR. However, the T allele failed to show any association with the risk of IUGR. In both the cases, the values could not reach the level of significance. Thus, the experiment should be done in more number of samples to reach any valid conclusion.

4. DISCUSSION-

Matrix metalloproteinases are implicated in ECM remodeling/ degradation both in normal contexts, such as placentation, and pathological contexts such as cancer. In embryo implantation, their actions allow trophoblast cells to invade maternal endometrium/myometrium but also maternal vessels, the latter in order to allow faster changes in blood flux. However, despite the important role of metalloproteinases in placentation, the possible association between functional MMP2 polymorphisms and IUGR has less investigated,

In our study, it was found that in IUGR cases 60% of infants had having CC genotype of MMP2 C>T SNP and 40% had CT genotype, while in control group 63.1% infants having CC genotype and 36.9% infants had CT genotype .

Genotypes, CT of MMP2 C>T polymorphism, shows a slight increase in risk towards the risk of IUGR. However, the T allele failed to show any association with the risk of IUGR.

In the IUGR population, MMP2 C> T transition was more frequent and risk of IUGR occurrence consequently increased in CT carriers, therefore showing an increased risk towards IUGR. In various studies, MMP2 C−1306T mutation have been found to decrease MMP2 promoter activity in epithelial cells, macrophages and smooth muscle cells [10]. If the mutation's effect is the same in trophoblast cells, foetuses carrying a T allele should indeed show decreased MMP2 protein

production, lower ECM degradation capacity and lower foetal cell penetration in maternal uterus, hence increasing the risk for IUGR occurrence.

This study adds a predictive factor, MMP2−1306 SNP, easily detectable early in pregnancy, to the growing list of risk factors for IUGR. It gives also a supplementary example of the contribution of the foetus to his or her own outcome, as already been shown for different cytokine polymorphisms in preterm delivery [11,12,13]. Indeed, foetuses carrying one or two T allele at position −1306 of the MMP2 gene have a higher risk risk (Odds Ratio >1)of IUGR than the normal CC genotype carriers. However, IUGR has a very complex pathology and this result needs to be added to the list of risk factors already identified to help define a more precise clinical, genotypic and biochemical profile of 'at risk' individuals for IUGR.

CONCLUSION-

This study suggests that there are several factors interplaying which lead IUGR babies. Sociodemographic factors like maternal age, educational level, socio economic status, nutritional status and anemia are very important. this study also suggests that polymorphism in cytokine gene may serve as protective role in general healthy population, however it is not consistent and may vary depending on ethnicity, phenotypic features and other environmental factors that characterize a particular population. Research to identify the influence of interaction among cytokine gene polymorphisms and environmental factors in various populations will help us to better understanding.

This study adds to a possible predictive factors such as MMP2−1306 C>T easily detectable early in pregnancy, to the growing list of risk factors for IUGR cases. Fetuses carrying one T at position −1306 of the MMP2 gene shows increased risk (Odds Ratio >1) of IUGR than the normal CC genotype carriers. However, FGR is a very complex pathology and this result is to be added to the list of risk factors already identified to help define a more precise clinical, genotypic and biochemical profile of 'at risk' individuals for IUGR.

In conclusion, the present study suggests that MMP2−1306 C>T gene polymorphisms can't modulate a woman's IUGR risk. We propose additional large and well-designed studies to investigate the genetic and environmental factors related to immunity and inflammation to predict IUGR risk.

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