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Original Research Article

Recurrent miscarriage in North Indian population: a study of association of polymorphisms in genes coding for the natural killer: cell receptor natural killer group 2, member D and its ligand MHC class I chain-related protein A

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ABSTRACT

Background: The objective of this present study was to investigate the possible association of natural killer group (NKG) receptors gene polymorphisms and MHC class I chain-related protein A (MICA) gene polymorphism with recurrent spontaneous abortion (RSA).
Methods: Three single-nucleotide polymorphism (SNPs) in NKG2D gene (rs2255336, rs2617160 and rs2617170) and one SNP in MICA gene (MICA129) rs1051792 were assessed in 100 controls and 100 patients employing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and agarose gel electrophoresis.
Results: NKG2D (rs2617160) and MICA 129 (rs1051792) variants are associated with RSA risk in North Indian women.
Conclusions: The NKG2D and MICA129 gene polymorphisms may influence the success of pregnancy in North Indian women population.

Keywords: MICA 129 gene, NKG2D gene, Single-nucleotide polymorphism, Unexplained recurrent spontaneous abortion

INTRODUCTION

Recurrent miscarriage (RM) or recurrent spontaneous abortion (RSA) is usually defined as the loss of three or more consecutive pregnancies until the 20th gestational week. Globally, 1-2% of reproductive couples are affected by RSA. The causes of a miscarriage can be genetic, structural deformities of organs, hormonal, immunological, haematological, and/or environmental, but in about half the cases cause may not be found.[1] NKG2D (Natural killer (NK) group 2, member D) is a 25,143 kDa type II membrane protein comprising 216 amino acids, is an activating receptor expressed on NK and CD8 T cells and has been implicated in immunity against tumors and microbial pathogens. To exert cytotoxicity, major histocompatibility complex class I polypeptide-related sequence (MIC) molecules interact with the NKG2-D receptor and stimulate NK cells to release cytokines such as interferon- (IFN). KLRK1 gene (killer cell lectin-like receptor of the subfamily K member 1), on chromosome 12, encodes NKG2D, or CD314. The NK cells play an important role in the maintenance of pregnancy. Decidual NK cells produce a variety of cytokines contributing to uterine vascular remodeling.[2] The maternal NK cells-mediated cytotoxicity limits the excessive trophoblast invasion

during the formation of the placenta. Thus, any imbalance between the activation and the regulation system in NK cells will alter the homeostasis at the feto-maternal interface and the fetal allograft during pregnancy leading to a miscarriage.[3] Negishi et al showed that NK cells (CD56dim CD16+NK cells) may play an important role in the onset of preterm labor.[4] MICA molecules are found expressed in the human endometrium, on epithelial cells in secretory phase of menstrual cycle.[5] MICA upregulation induces maternal rejection of fetus, in response to stresses of pregnancy such as hypoxia or aneuploidy.[6] Expression of MICA molecules mRNA was detected in trophoblast and decidual cells, but no protein expression demonstrated, on implantation site at 8 weeks gestation cells from normal pregnancy.[7]

METHODS

This study was descriptive case control study. The study was conducted over a period of two years from January 2017 to December 2018.

The study group consisted of 100 North Indian women of reproductive age group diagnosed with RSA, who attended as outpatients in medical genetics, for genetic evaluation and counselling of recurrent spontaneous abortions. All patients had at least three pregnancy losses with unexplained etiology before 20th week of gestation.

The control group consisted of 100 ethnically matched women (reproductive age group, no history of autoimmune disorder, no known personal or family history of RSA or other pregnancy complications) with at least one normal pregnancy and no spontaneous abortion, preterm labour, or preeclampsia.

Inclusion criteria

In woman of reproductive-age group, who had at least three pregnancy losses before 20th week of gestation, the diagnosis of RSA was made.

Exclusion criteria

Any of the woman who matched any of the criteria as mentioned below was excluded from the study;

Chromosomal abnormalities of recurrent abortions in couple were excluded by karyotype of couple. Anatomic causes including intrauterine malformations, uterine fibroids and intrauterine adhesions (Asherman's syndrome) were excluded by pelvic examination and ultrasound. Hormonal causes such as hyperprolactinaemia, luteal insufficiency and hyperandrogenaemia were excluded. Autoimmune and thrombotic causes such as lupus and antiphospholipid antibody syndrome were excluded by evaluating lupus anticoagulant, anticardiolipin antibodies and anti-beta-2 glycoprotein. Medical causes such as thyroid disorders, diabetes mellitus, polycystic ovarian syndrome and systemic lupus erythematosus were excluded.

Consent and sample collection

Objectives of the study were explained to the couple and written informed consent was obtained from each individual for collection of clinical information and peripheral blood samples. Similar data collection procedures were used for patients and control subjects. Genetic study was performed on all patients and control subjects. Venous blood samples were collected in EDTA-containing tubes.

Laboratory methods

DNA extraction

Genomic DNA extraction was done from EDTA blood using the QIAamp DNA blood mini kit (Qiagen) method.[8]

Methodology for SNP analysis

As NKG2D gene (rs2617160) and MICA 129 (rs1051792) harbored a restriction site hence analysis was performed by PCR-restriction fragment length polymorphism (RFLP) as per feasibility and cost effectiveness. While for SNP rs2617170 in NKG2D gene, there was no restriction site; so, detection was done by Q-RTPCR.

SNP in NKG2D gene (rs: 2617170): T/A

Genotyping was performed by a TaqMan 5-nuclease assay (applied biosystems) with allele specific fluorogenic oligonucleotide probes for NKG2D alleles, allowing to discriminating the genotypes of each studied pair of alleles.

Table 1: Itinerary for restriction digestion NKG2D gene (rs2617160).[10]

Restriction enzyme (ScaI)	1.0 micro L (10 Unit)
PCR product	15.0 micro L
10X NE buffer	2.5 micro L
MQ	6.5 micro L
Total reaction volume	25.0 micro L
Incubation time	Overnight at 37ºC

Genotyping was performed by the allelic discrimination method using VIC- and FAM-labelled primers. The reaction was performed in 20 microlitre (microL) as recommended by the manufacturer, 10 micro L Taq Man Universal master mix, 1 µL FAM and VIC labelled primer, (Forward and Reverse) and 1 microL DNA.[9] Thermal cycling was initiated with a 2-min incubation at 50ºC, followed by a first denaturation step of 10 min at 95ºC, and then by 40 cycles of 15 s at 95ºC and of 1 min

at 60ºC. PCR plates were run and read in the ABI prism 7000 sequence detection system (applied biosystems). Results were analysed using the allelic discrimination software.[10]

SNP in NKG2D gene (rs2617160): T/A

SNP in NKG2D gene (rs2617160) was analysed using the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method. The primers were as follows: 5' - GTCGTTAAAGGCATCGTTCC - 3' (forward) and 5' - GACTTAACACGCAGCCAACT - 3' (reverse) for the PCR of NKG2D.[11]

Table 2: Itinerary for restriction digestion MICA 129 (rs1051792).[12]

Restriction enzyme (Rsa)	1.0 micro L (10 Unit)
PCR product	15.0 micro L
10X NE buffer	2.5 micro L
MQ	6.5 micro L
Total reaction volume	25.0 micro L
Incubation time	Overnight at 37ºC

The PCR product of 1051 base pair was digested with ScaI restriction endonuclease (New England Biolab) at 37ºC for overnight. The digested PCR products were analysed on 3% agarose gel electrophoresis for detection of SNP. Authors found TT homozygous wild type 1,051 bp, TA heterozygous mutant 1,051, 580 and 471 bp and AA homozygous mutant 580 and 471 bp. The gel was stained with ethidium bromide and image was captured in geldoc where UV transilluminator visualized the bands.[10]

MICA 129 genotyping (rs1051792): A/G = Met/Val

The MICA 129 polymorphism (rs1051792) was analysed using the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method. The primers were as follows: 5' -CGTTCTTGTCCCTTTGCCC GTGTGC - 3' (forward; intron 1) and 5' -GATGCTG CCCCCATTCCCTTCCCAA - 3' (reverse; intron 5) for the first PCR, and 5' -GGGTCTGTGAGATCCATGA-3(forward; exon 3) and 5' – TGAGCTCTGGAG GACTGGGTA-3' (reverse; exon 3) for the second PCR. The reverse primer for the second PCR step was modified to create a Rsa I recognition site, so that the MICA 129Val sequence could be identified with Rsa I.[12]

Nested PCR products of MICA gene (122 bp) was digested with RsaI restriction endonuclease (New England Biolab) at 37◦C for overnight. The digested PCR products were analysed on 3% agarose gel electrophoresis for detection of SNP. Authors find in MICA-129 met/met wild type homozygous 127 bp, in MICA-129 met/val mutant type heterozygous 127, 104 and 23 bp and in MICA-129 val/val mutant type homozygous 104 bp and 23 bp on gel. The gel was stained with Ethidium bromide and image was captured in geldoc where UV transilluminator visualized the bands.

Statistical analysis

Statistical analysis was performed on SPSS v.17.0 [13]. Pearson's chi-square test was used to assess intergroup significance (categorical variables). Gene polymorphisms were analysed for allelic frequency for both patients and controls.

RESULTS

Patients and controls

A total of hundred study subjects who had three or more miscarriages and hundred controls were included in the study.

Genetic polymorphisms three SNPs in NKG2D gene (rs2255336, rs2617160 and rs2617170) and one SNP in MICA gene (MICA129) rs: 1051792 taken for this study were assessed in 100 controls and 100 patients employing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and agarose gel electrophoresis.

Table 3: Allele and genotypic frequencies of NKG2D gene (rs:2617170) T/A) in Indian population.

	Patients	Controls	Odds ratio (95% CI)	P-value
Total women	**n=100 (patients)**	**n=100 (control)**		
Allele frequency (total number of alleles)				
C	0.52 (105)	0.54 (108)	1.0 (reference)	
T	0.48 (95)	0.46 (92)	0.598 (0.31 - 1.138)	0.764
Genotypic frequency (total number of genotypes)				
C/C	0.35 (35)	0.26 (26)	1.0 (reference)	
C/T	0.45 (45)	0.56 (56)	0.598 (0.312-1.138)	0.118
T/T	0.20 (20)	0.18 (18)	0.827 (0.362-1.885)	0.65
C/T + T/T	0.65 (65)	0.74 (74)	0.653 (0.353-1.201)	0.171

One SNP in NKG2D gene (rs2617170) were assessed by allelic discrimination (real-time PCR) in both patients and control women. In this study authors investigated the possible association of natural killer group (NKG) receptors gene polymorphisms and MHC class I chain-related protein A (MICA) gene polymorphism with RSA.

Allelic and genotypic frequency C/T in NKG2D gene (rs: 2617170)

NKG2D gene (rs: 2617170) polymorphism [OD ratio for carriers of the T allele = 0.598; 95%; CI = (0.31-1.138), p value; 0.764 and OD ratio for carriers of the C/T allele = 0.598; 95%; CI= (0.312-1.138), p value; 0.118]. The OD ratio for combined homozygous mutant (TT) and heterozygous mutant type C/T with reference to homozygous C/C is 0.653, 95% CI = (0.353-1.201) and p value of 0.171. The findings did not exhibit statistically significant association with RSA in females and controls in North Indian population (Table 3).

Allelic and genotypic frequency T/A in NKG2D gene (rs2617160)

In this study authors found that NKG2D gene (rs2617160) carrier T/A heterozygous polymorphism [(OD ratio for carriers of the T/A allele = 2.342; 95%; CI = (1.27-4.35), p value; 0.006] showed significant association with RSA risk in Indian female population (Table 4). Homozygous A/A polymorphism [(OD ratio for carriers of the A/A allele = 2.694; 95%; CI = (1.084-6.917), p value; 0.032] was significantly associated with disease. Also, there is significant association in combined heterozygous mutant and homozygous mutant (T/A+A/A) with OD ratio 2.418 (95% CI = (1.35-4.35) and p value is 0.002 (Table 4). This confirmed that homozygous A/A, heterozygous T/A and combined heterozygous T/A and homozygous mutant A/A were significantly associated with RSA risk in North Indian population.

Table 4: Allele and genotypic frequencies of NKG2D gene (rs2617160) T/A) in Indian population.

	Patients	Controls	Odds ratio (95% CI)	P-value
Total women	**n=100 (patients)**	**n=100 (controls)**		
Allele frequency (total number of alleles)				
T	0.57 (114)	0.64 (141)	1.0 (reference)	
A	0.43 (86)	0.36 (59)	1.8 (1.192-2.73)	0.0051
Genotypic frequency (total number of genotypes)				
T/T	0.28 (30)	0.45 (51)	1.0 (reference)	
T/A	0.58 (54)	0.39 (39)	2.342 (1.27-4.35)	0.006
A/A	0.14 (16)	0.16 (10)	2.694 (1.084-6.917)	0.032
T/A+ A/A	0.71 (70)	0.55 (49)	2.418 (1.35-4.35)	0.002

Table 5: Allele and genotypic frequencies of MICA 129 polymorphism (rs1051792) A/G) in North Indian population.

	Patients	Controls	Odds ratio (95% CI)	P-value
Total women	**n=100 (patients)**	**n=100 (controls)**		
Allele frequency (total number of alleles)				
A	0.5 (101)	0.63 (126)	1.0 (reference)	
G	0.5 (99)	0.37 (74)	1.667 (1.119-2.49)	0.011
Genotypic frequency (total number of genotypes)				
A/A	0.26 (26)	0.44 (44)	1.0 (reference)	
A/G	0.49 (49)	0.38 (38)	2.171 (1.142-4.17)	0.017
G/G	0.25 (25)	0.18 (18)	2.593 (1.056-6.522)	0.032
A/G + G/G	0.74 (74)	0.56 (56)	2.227 (1.229-4.08)	0.008

Allelic and genotypic frequency A/G in MICA 129 genotyping (rs1051792)

MICA 129 (rs1051792) carrier A/G heterozygous polymorphism [(OD ratio for carriers of the A/G allele = 2.171; 95%; CI = ((1.142-4.17), p value; 0.017] showed significant association with RSA risk in North Indian female population (Table 5). Authors calculated the OD ratio and p value for homozygous G/G mutant genotype and found significant association with RSA, G/G homozygous polymorphism [(OD ratio for carriers of the G/G allele = 2.593; 95%; CI = (1.056-6.522), p value; 0.032]. Also, there was significant association in combined heterozygous mutant and homozygous mutant

(A/G+G/G). A/G and G/G has OD ratio 2.227, 95% CI = (1.229-4.08) and p value is 0.008 (Table 5). This confirmed that homozygous mutant G/G, heterozygous mutant A/G and combined (G/G and A/G) were significantly associated with RSA risk in North Indian population (Table 5).

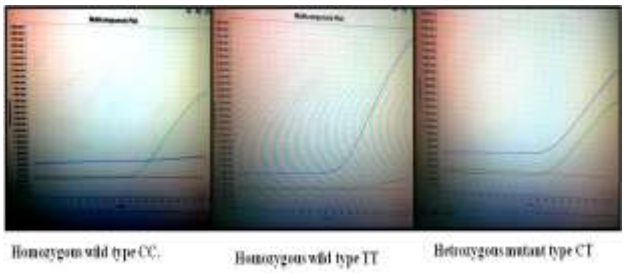

Figure 1: Allele discrimination on the basis of amplification plot (Vic and Fam).

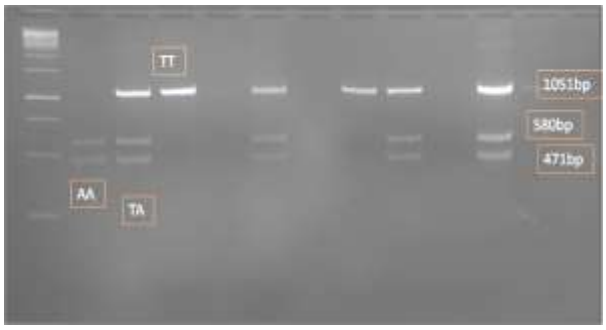

Figure 2: Detection of SNP in NKG2D (rs2617160) in 2% agarose gel.

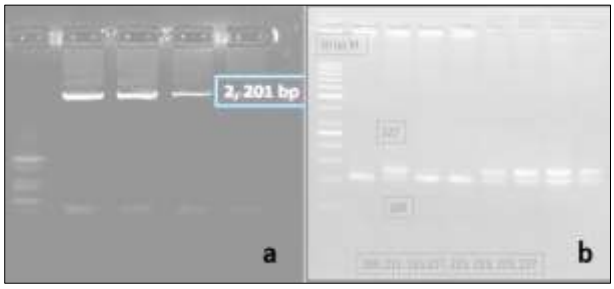

Figure 3: a) Detection of first PCR product (2201 bp), b) Detection of digested nested PCR product with Rsa I on 3% agarose gel.

DISCUSSION

Various causes have been implicated in the etio-pathogenesis of RSA, apart from immunological factors, Genetic polymorphisms in various genes is a subject of research. Rad et al found increased NK cells in the peripheral blood of women with recurrent miscarriages.[14] NKG2D plays an important role through cytotoxicity and cytokine release in the pathogenesis of infections, autoimmune diseases, and GVHD.[15-17]

Hayashi et al, and Espinoza et al found association between NKG2D haplotype and phenotype (high or low natural cytotoxic activity) in the occurrence of cancer and the transplants outcomes. rs2617160 and rs2617170, were significantly associated with reduced risk of cancer and better clinical outcome after transplantation.[18,19] In the Tunisiancohort of Hizem et al T/T genotype in SNP rs2617170 was associated with a higher cytotoxic activity and a decreased risk of cancer development, while it was associated with successful pregnancy.[10] Authors found there was no statistically significant association of NKG2D gene (rs: 2617170) polymorphism in RSA in females and controls in North Indian population. This was in contrast to Hizem et al who found significant difference in mean allele frequency (MAF) of NKG2D 11 (rs2617170) between patients and controls (p=0.04) that translated into a low protective effect against RSA in the Tunisian population [OR (95%) = 0.79 (0.64-0.99)].[10] This could be due to ethnicity, environmental factors or modifier effect of other variants.

Various studies have shown that TT genotype in rs2617160 is associated with increased risk of SLE and chronic hepatitis B due to high cytotoxic activity.[20,21] Authors found that NKG2D gene (rs2617160) carrier T/A heterozygous polymorphism [(OD ratio for carriers of the T/A allele = 2.342; 95%; CI = (1.27-4.35), p value; 0.006] showed significant association with RSA risk in north Indian female population which could as well be due to immunomodulation effect of the heterozygous T/A genotype.

This MICA-129 polymorphism (rs1051792) has been associated with a number of diseases such as ankylosing spondylitis, cancer, and chronic graft-versus-host disease (cGVHD).[22-24] It was suggested that a potential cytotoxic attack by maternal NK was the down-regulation of the activating NK-cell receptor NKG2D by the soluble MIC molecules (sMICA) which results from the proteolytic shedding of the membrane-bound molecules by placental exosomes.[25,26] While authors found MICA 129 (rs1051792) carrier A/G heterozygous polymorphism [(OD ratio for carriers of the A/G allele = 2.171; 95%; CI = (1.142-4.17), p value; 0.017] showed significant association with RSA risk in North Indian female population. Linsingen et al in their study found, MICA 129 Met/Val dimorphism in mother and sMICA plasma levels did not differ between cases and controls and that MICA-129Val/Val genotype was associated with higher sMICA plasma levels.[27] While Hizem et al found no significant association with RSA for MICA-129 (rs1051792).[10]

To the best of authors knowledge this is the first such study to be reported from the north Indian female population. The contrasting results in this study as compared to Tunisian population Hizem et al could be explained due to modifier effect of variants in other genes influencing the cytokine release, cytotoxicity and

immunomodulation, however study on a larger Indian cohort is required.

CONCLUSION

In conclusion, this study data supports an effect of the NKG2D (rs2617160) and MICA 129 (rs1051792) variants on RSA risk in North Indian women. This association of NKG2D and MICA 129 genetic variants to RSA risk should be viewed in context of the cytokine environment in pregnancy along with modifier effects of other polymorphisms in NKG2D and other genes. Further studies on a larger population are needed to confirm the current findings and to reveal the underlying mechanism by which NKG2D polymorphisms influence overall RSA risk.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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