

# KIR Gene Frequencies in Women with Infertility Problems

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## Abstract

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**BACKGROUND:** Natural killer (NK) cells are the predominant lymphocyte population in the decidua. Being the most abundant leucocytes, the activity of NK cells is important in different immuno-pathological conditions, such as recurrent spontaneous abortions, infertility and problems in implantation. The NK cells recognize HLA class I molecules on trophoblasts through killer immunoglobulin-like receptors (KIRs) found on their surface. The KIRs are classified as either activating or inhibitory, regarding the effect they produce on NK cells upon interaction with corresponding ligand. Since KIR genes exhibit extensive polymorphism and individuals differ in both the number and kind (activating vs. inhibitory) of KIR genes, it is hypothesized that the KIR gene content might influence the pregnancy outcome.

**AIM:** The aim of this pilot study is to analyze the frequency of different KIR genes in women with infertility problems, and compare them to healthy women.

**MATERIAL AND METHODS:** Total of 122 healthy women (Control) and 25 women with reproductive problems (MISSC) participated in this study. After signing of written consent DNA was isolated from peripheral blood using phenol/chloroform method. The genotyping of 16 KIR genes was performed using commercially available kit from Dynal Biotech, (Pel-Freez Clinical Systems, Brown Deer, WI, USA), based on SSP method.

**RESULTS:** We found that inhibitory KIR are present in similar observed frequency in both control and patients with MISSC, except KIR2DL5 which was found in lower frequency in patients with MISSC. There are no significant differences of all noninhibitory KIR between control and patients with MISSC. The number of inhibitory KIR genes in patients with MISSC was lower, except for seven inhibitory KIR genes which was almost doubled. The number of noninhibitory (stimulatory) KIR genes was lower in patients with MISSC, except for those with three KIR genes which were almost four times more frequent. We found significantly bigger percentage of 0.34 – 0.60 activating/inhibitory KIR gene number ratio in the patients with MISSC.

**CONCLUSION:** In conclusion, there are differences in the KIR gene distribution, gene number, and activating/inhibitory KIR gene number ratio between control and Macedonian patients with MISSC. Further analysis of frequencies of corresponding KIR genotypes or in the ratio of activating/inhibiting genes content in two groups are needed.

## Introduction

Natural killer (NK) cells are the predominant lymphocyte population in the decidua. Being the most abundant leucocytes, the activity of NK cells is important in different immuno-pathological conditions, such as recurrent spontaneous abortions, infertility and problems in implantation. The NK cells recognize HLA class I molecules on trophoblasts through killer immunoglobulin-like receptors (KIRs) found on their

surface. The KIRs are classified as either activating or inhibitory, regarding the effect they produce on NK cells upon interaction with corresponding ligand. Since KIR genes exhibit extensive polymorphism and individuals differ in both the number and kind (activating vs. inhibitory) of KIR genes, it is hypothesized that the KIR gene content might influence the pregnancy outcome.

Natural killer (NK) cell receptors (NKR) have been suggested to protect trophoblast, but their function at the fetomaternal interface remains

unknown. To investigate if the outcome of pregnancy depends on women's NKRs, we studied the NKR repertoire in couples with recurrent spontaneous abortions (RSA). Less aborters than controls were found to have all three inhibitory KIRs, some of them had only one inhibitory KIR and most of them were lacking inhibitory KIRs possessed by their husbands. It was concluded that women with alloimmune abortions have a limited inhibiting KIR repertoire and such miscarriages may occur because trophoblastic HLA class I molecules are recognized by decidual NK cells lacking the appropriate inhibitory KIRs [1].

Another study from a total population of 158 recurrent spontaneous abortion (RSA) couples, 40 couples with repeated implantation failures (IVF) and 81 control couples, reported by five different laboratories, analysis was performed. RSA couples were divided into alloimmune aborter (RSAallo) and autoimmune aborter (RSAauto). The ratio of inhibitory to activating KIR (actKIR) was slightly lower in RSAallo and IVF women, while in a high percentage of these women, the standard receptors of the KIR A haplotype were combined with actKIR/s of the haplotype B. This may suggest a possible involvement of actKIRs in embryo implantation and the maintenance of pregnancy and also requires further investigation [2].

Published results from China showed that gene frequency of KIR2DS1 was higher in patients with RSA compared to that of control subjects. Increased numbers of activating KIR genes was observed in patients. Women who possessed more than two activating KIR genes were found more frequently in patients than those in control subjects. From a cohort of husband and wife couples, the women with a KIR2DS1 gene, and with a decreased group 2 HLA-C allele for the homologous inhibitory receptor KIR2DL1, had a tendency to fall into the RSA group. The results suggest that a genetic variation at the KIR locus influences the susceptibility to unexplained RSA in the Chinese Han population. Moreover, decreased ligands for inhibitory KIRs could potentially lower the threshold for NK cell activation, mediated through activating receptors, thereby contributing to pathogenesis of RSA [3].

Hiby et al., 2008 obtained DNA from the male and female partners of couples with three or more spontaneous miscarriages and genotyped for HLA-C groups and 11 KIR genes using the PCR-sequence-specific primer method (SSP). The frequency of the HLA-C2 group was increased in both parents compared with a parous control population. The KIR gene frequencies of the male partners were similar to controls, but the women had a high frequency of KIR AA haplotypes that lack activating KIR. In particular, the activating KIR for HLA-C2 groups (KIR2DS1) was significantly lower in these women. This was the first report to identify a genetic male factor that confers risk in RM. These findings support the idea that successful placentation depends on the correct balance of NK

cell inhibition and activation in response to trophoblast [4].

The aim of this pilot study is to analyze the frequency of different KIR genes in women with infertility problems, and compare them to healthy women.

## Methods

### Investigated groups

The total studied sample consists of 147 examinees composed of two different groups: healthy individuals (n=122) (Control) and patients with infertility problems (including spontaneous miscarriages) (MISSC) (n=25).

Each individual was interviewed on a one-to-one basis; his/her genealogy was recorded for the last three generations. Admixture, if any, was recorded for each individual. Individuals with only one Albanian parent were excluded from the study. After signing of written consent, genomic DNA was extracted from the peripheral blood leukocytes using standard phenol/chloroform procedure, described elsewhere [5], and stored in the anthropology project field of the Macedonian Human DNA Bank (hDNAMKD) [6] until processing.

### PCR amplification

For KIR genotyping, commercially available PEL-FREEZ KIR genotyping SSP kit (DynaL Biotech, Brown Deer, WI) was used. It is a PCR-based method (using sequence-specific priming approach) designed to detect the presence or absence of 16 KIR genes and pseudogenes defined by the International nomenclature committee of WHO [7, 8]. In brief, locus specific primer sets, dispensed in a 96 well thermal tray were used for amplification of genomic DNA. After the amplification, the PCR products are loaded and separated by electrophoresis onto a 2% agarose gel stained with ethidium bromide, after which the results are interpreted using a worksheet for the specific amplification patterns. The presence of each KIR gene was determined by the presence of a band of DNA of the expected size (Fig. 1).

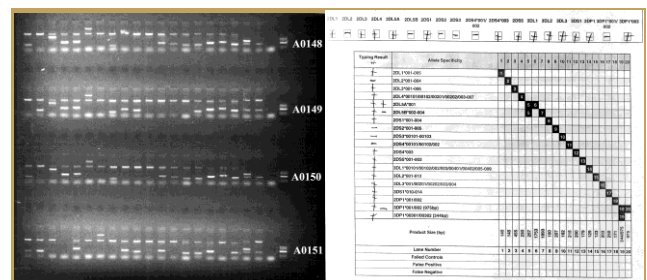


Figure 1: Electrophoresis of PCR products (left) and KIR genotyping documentation form (right).

All PCRs contained an internal positive control consisting of an additional pair of primers specific for the growth hormone (GH) gene and a negative control [9]. Individuals were determined negative for a particular *KIR* gene when a band of expected size was absent in the presence of a band for the GH gene. We have used external quality control consisting of cell lines from Immunogenetics and Histocompatibility Workshop Conferences and Centre d' Etude du Polymorphisme Humain.

### Statistical analysis

The occurrence of *KIR* genes in individuals (frequency = F) was obtained by direct counting. Gene frequencies (GF) were calculated using the formula  $GF=1-\sqrt{1-F}$ , being aware of the limitation in its ability to detect *KIR* genes present at low frequency. For analysis of the molecular polymorphism of the locus studied, the Arlequin software version 3.0 [10] was used.

Linkage disequilibrium (LD) values for two locus associations were calculated using 2x2 tables [11]. Because LD is not independent of allele frequencies, normalized LD was calculated as described previously [12, 13]. Comparisons of different genotypes for two groups were tested by the  $\chi^2$  test. Crude odds ratios (OR) were calculated within 95% CI.

## Results

Observed and estimated *KIR* gene frequencies for 25 women with infertility problems (including recurrent spontaneous miscarriages) (MISSC) and 122 healthy controls are shown in Table 1. We can see that *KIR* pseudogenes are present in all investigated women, except in one of the controls and one in the group with MISSC. Inhibitory *KIR* are present in similar observed frequency in both control and patients with MISSC, except *KIR2DL5* which was found in lower frequency in patients with MISSC (0.41 versus 0.16, respectively; Pearson's  $P = 0.018$ ). The most frequent noninhibitory *KIR* was *KIR2DS4* with estimated frequency of 0.80 in controls and 1 in patients with MISSC followed by *KIR2DS2* with the estimated frequency of 0.35 in controls and 0.34 in women with MISSC. There are no significant differences of all noninhibitory *KIR* between control and patients with MISSC.

The content of inhibitory and noninhibitory genes in controls and women with infertility problems is shown in Table 2. The number of inhibitory *KIR* genes in patients with MISSC was lower, except for seven inhibitory *KIR* genes which was almost doubled. The number of noninhibitory (stimulatory) *KIR* genes was lower in patients with MISSC, except for those with three *KIR* genes which were almost four times more frequent.

**Table 1: Observed and estimated *KIR* gene frequencies for 25 women with infertility problems (including recurrent spontaneous miscarriages) (MISSC) and 122 healthy controls.**

	Pseudogenes		Inhibitory <i>KIR</i>									Noninhibitory <i>KIR</i>					
	<i>KIR</i> 2DP1	<i>KIR</i> 3DP1	<i>KIR</i> 2DL1	<i>KIR</i> 2DL2	<i>KIR</i> 2DL3	<i>KIR</i> 2DL4	<i>KIR</i> 2DL5	<i>KIR</i> 3DL1	<i>KIR</i> 3DL2	<i>KIR</i> 3DL3	<i>KIR</i> 2DS1	<i>KIR</i> 2DS2	<i>KIR</i> 2DS3	<i>KIR</i> 2DS4	<i>KIR</i> 2DS5	<i>KIR</i> 3DS1	
MISSC	OF	0.96	1	0.96	0.68	0.96	0.96	0.16 *	0.96	1	1	0.32	0.56	0.44	1	0.24	0.28
	S.D. of	0.04	0	0.04	0.095	0.04	0.04	0.075	0.04	0	0	0.095	0.101	0.101	0	0.087	0.092
	EF	0.80	1	0.80	0.43	0.80	0.81	0.08	0.80	1	1	0.18	0.34	0.25	1	0.13	0.15
Control	OF	0.98	1	0.94	0.59	0.90	1	0.41 *	0.96	1	1	0.48	0.57	0.33	0.96	0.30	0.38
	S.D. of	0.01	0	0.02	0.04	0.03	0	0.04	0.018	0	0	0.045	0.04	0.04	0.018	0.04	0.04
	EF	0.87	1	0.76	0.36	0.69	1	0.23	0.80	1	1	0.28	0.35	0.18	0.80	0.17	0.22

MISSC, women with infertility problems (including recurrent spontaneous miscarriages); \* Pearson's  $P = 0.018$ ; OR = 0.274; WALD 95% CI = 0.089 – 0.848.

The ratios of number of activating and inhibitory *KIR* genes in control and patients with MISSC are shown in Table 3. Activating/inhibitory *KIR* gene number ratio was classified in three groups: those with ratio less than 0.33; ratio between 0.34 and 0.60; and ratio above 0.60. Significantly bigger percentage of 0.34 – 0.60 ratio was found in the patients with MISSC (Pearson's  $P = 0.035$ ; OR = 0.398; WALD 95%CI = 0.166 – 0.956).

**Table 2: The content of inhibitory and noninhibitory (activating) genes in controls and women with infertility problems.**

Group	Number of inhibitory <i>KIR</i> genes					Number of activating <i>KIR</i> genes					
	4	5	6	7	8	1	2	3	4	5	6
Control (N=122)	0.8 (1)	1.6 (2)	38.5 (47)	34.4 (42)	24.6 (30)	24.6 (30)	19.7 (24)	9.8 (12)	26.2 (32)	13.1 (16)	6.5 (8)
MISSC (N=25)	0	4.0	32.0	56.0	8.0	12.0	28.0	36.0	12.0	12.0	0

**Table 3: Ratios of number of noninhibitory (activating) and inhibitory KIR genes.**

Group	Activating/inhibitory KIR gene number ratio (number)		
	< 0.33	0.34 – 0.60	> 0.60
Control (N=122)	43.4 (53)	33.6 (41) *	23.0 (28)
MISSC (N=25)	32.0 (8)	56.0 (14) *	12.0 (3)

\* Pearson's P = 0.035; OR = 0.398; WALD 95% CI = 0.166 – 0.956

## Discussion

In this pilot study we present differences in the KIR gene frequencies between control and patients with MISSC. We found that inhibitory KIR are present in similar observed frequency in both control and patients with MISSC, except KIR2DL5 which was found in lower frequency in patients with MISSC. There are no significant differences of all noninhibitory KIR between control and patients with MISSC. The number of inhibitory KIR genes in patients with MISSC was lower, except for seven inhibitory KIR genes which was almost doubled. The number of noninhibitory (stimulatory) KIR genes was lower in patients with MISSC, except for those with three KIR genes which were almost four times more frequent. We found significantly bigger percentage of 0,34 – 0,60 activating/inhibitory KIR gene number ratio in the patients with MISSC.

In the frame of International HLA and Immunogenetics Workshop there is reproductive immunology component which summarize the results from several scientific groups dealing with KIR and recurrent spontaneous abortion (RSA). They published several papers in which investigate if the outcome of pregnancy depends on women's NKR's. They concluded that women with alloimmune abortions have a limited inhibiting KIR repertoire and such miscarriages may occur because trophoblastic HLA class I molecules are recognized by decidual NK cells lacking the appropriate inhibitory KIRs [1]. In another study they performed analysis from a total population of 158 recurrent spontaneous abortion (RSA) couples, 40 couples with repeated implantation failures (IVF) and 81 control couples, reported by five different laboratories. RSA couples were divided into alloimmune aborter (RSAallo) and autoimmune aborter (RSAauto). It was suggested a possible involvement of actKIRs in embryo implantation and the maintenance of pregnancy [2].

Published results from China suggest that a genetic variation at the KIR locus influences the susceptibility to unexplained RSA in the Chinese Han population. Moreover, decreased ligands for inhibitory KIRs could potentially lower the threshold for NK cell activation, mediated through activating receptors, thereby contributing to pathogenesis of RSA [3]. Hiby et al., 2008 reported for the first time that genetic male factor confers risk in RM. These findings support the idea that successful placentation depends on the

correct balance of NK cell inhibition and activation in response to trophoblast [4]. Several studies suggest that the imbalance of inhibitory and activating KIRs in uterine NKs might confer susceptibility to the occurrence of pregnancy loss. The maternal inhibitory/activating KIRs-HLA-C polymorphism expressed on trophoblast cells from decidual tissues seems to play a limited role in abortion [14-17]. In addition, the results suggest for the first time that sporadic and recurrent spontaneous abortions as well as miscarriage in the presence or absence of autoantibodies may have different KIR genotypic backgrounds [18].

The results from the 15th International Histocompatibility Workshop oppose the suggestion that increased HLA-DQA1\*0505 sharing predispose to RSA or RIF. The KIR2DL3-C1 combination (or lack of the KIR2DL1-C2 one) is associated with implantation failure [19].

Several papers published association of HLA\**C* with KIR in patients with MISSC. They suggest that among KIR AA women who have HLA-C C2C2 partners, HLA-C heterozygous females show a trend towards an increased chance of successful pregnancy [20-22].

In conclusion, there are differences in the KIR gene distribution, gene number, and activating/inhibitory KIR gene number ratio between control and Macedonian patients with MISSC. Further analysis of frequencies of corresponding KIR genotypes or in the ratio of activating/inhibiting genes content in two groups are needed.

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## References

1. Varla-Leftherioti M, Spyropoulou-Vlachou M, Niokou D, Keramitsoglou T, Darlamitsou A, Tsekoura C, Papadimitropoulos M, Lepage V, Balafoutas C, Stavropoulos-Giokas C. Natural killer (NK) cell receptors' repertoire in couples with recurrent spontaneous abortions. *Am J Reprod Immunol.* 2003;49(3):183-91.
2. Varla-Leftherioti M, Keramitsoglou T, Spyropoulou-Vlachou M, Papadimitropoulos M, Kontopoulou-Antonopoulou V, Tsekoura C, Sankarkumar U, Paparistidis N, Ghosh K, Pawar A, Vrani V, Daniilidis M, Parapanissiou E, Diler AS, Carin M, Stavropoulos-Giokas C. 14th International HLA and Immunogenetics Workshop: report from the reproductive

- immunology component. *Tissue Antigens*. 2007;69 Suppl 1:297-303.
3. Wang S, Zhao YR, Jiao YL, Wang LC, Li JF, Cui B, Xu CY, Shi YH, Chen ZJ. Increased activating killer immunoglobulin-like receptor genes and decreased specific HLA-C alleles in couples with recurrent spontaneous abortion. *Biochem Biophys Res Commun*. 2007;360(3):696-701.
  4. Hiby SE, Regan L, Lo W, Farrell L, Carrington M, Moffett A. Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage. *Hum Reprod*. 2008;23(4):972-6.
  5. Towner P. Purification of DNA. *Essential Molecular Biology*. Ed by Brown TA. Oxford, Oxford University Press, 1995: 47-54.
  6. Spiroski M, Arsov T, Petlichkovski A, Strezova A, Trajkov D, Efinska-Mladenovska O, Zaharieva E. Case Study: Macedonian Human DNA Bank (hDNAMKD) as a source for public health Genetics. *Health Determinants in the Scope of New Public Health*. B. G. Georgieva L. Sofia, Hans Jacobs, 2005.
  7. Marsh SG, Parham P, Dupont B, Geraghty DE, Trowsdale J, Middleton D, Vilches C, Carrington M, Witt C, Guethlein LA, Shilling H, Garcia CA, Hsu KC, Wain H. Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics*. 2003;55(4):220-6.
  8. Robinson J, Mistry K, McWilliam H, Lopez R, Marsh SG. IPD--the Immuno Polymorphism Database. *Nucleic Acids Res*. 2010;38(Database issue):D863-9.
  9. Gómez-Lozano N, Vilches C. Genotyping of human killer-cell immunoglobulin-like receptor genes by polymerase chain reaction with sequence-specific primers: an update. *Tissue Antigens*. 2002;59(3):184-93.
  10. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol Bioinform Online*. 2007;1:47-50.
  11. Mattuiz PL, Ihde D, Piazza A, Ceppellini R, Bodmer WF. New approaches to population genetics and segregation analysis of the HLA system. In: TerasakiPI, editor. *Histocompatibility testing*. Copenhagen: Munksgaard; 1970. p. 193–205.
  12. Lewontin RC, Dunn LC. The Evolutionary Dynamics of a Polymorphism in the House Mouse. *Genetics*. 1960;45(6):705-22.
  13. Lewontin RC. The Interaction of Selection and Linkage. I. General Considerations; Heterotic Models. *Genetics*. 1964;49(1):49-67.
  14. Hong Y, Wang X, Lu P, Song Y, Lin Q. Killer immunoglobulin-like receptor repertoire on uterine natural killer cell subsets in women with recurrent spontaneous abortions. *Eur J Obstet Gynecol Reprod Biol*. 2008;140(2):218-23.
  15. Faridi RM, Das V, Tripathi G, Talwar S, Parveen F, Agrawal S. Influence of activating and inhibitory killer immunoglobulin-like receptors on predisposition to recurrent miscarriages. *Hum Reprod*. 2009;24(7):1758-64.
  16. Vargas RG, Bompeixe EP, França PP, Marques de Moraes M, da Graça Bicalho M. Activating killer cell immunoglobulin-like receptor genes' association with recurrent miscarriage. *Am J Reprod Immunol*. 2009;62(1):34-43.
  17. Moffett A, Hiby S. Influence of activating and inhibitory killer immunoglobulin-like receptors on predisposition to recurrent miscarriages. *Hum Reprod*. 2009;24(8):2048-9.
  18. Nowak I, Malinowski A, Tchorzewski H, Barcz E, Wilczynski JR, Grybos M, Kurpisz M, Luszczek W, Banasik M, Reszczyńska-Slezak D, Majorczyk E, Wisniewski A, Senitzer D, Yao Sun J, Kusnierczyk P. Frequencies of killer immunoglobulin-like receptor genotypes influence susceptibility to spontaneous abortion. *J Appl Genet*. 2009;50(4):391-8.
  19. Varla-Leftherioti M, Keramitsoglou T, Parapanissiou E, Kurpisz M, Kontopoulou-Antonopoulou V, Tsekoura C, Kamienczna M, Novokowska B, Papanistidis N, Vrani V, Daniilidis M, Spyropoulou-Vlachou M. HLA-DQA1\*0505 sharing and killer immunoglobulin-like receptors in sub fertile couples: report from the 15<sup>th</sup> International Histocompatibility Workshop. *Tissue Antigens*. 2010;75(6):668-72.
  20. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, Claas FH, Walker JJ, Redman CW, Morgan L, Tower C, Regan L, Moore GE, Carrington M, Moffett A. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest*. 2010;120(11):4102-10.
  21. Nowak I, Malinowski A, Tchorzewski H, Barcz E, Wilczyński JR, Banasik M, Gryboś M, Kurpisz M, Luszczek W, Majorczyk E, Wiśniewski A, Senitzer D, Sun JY, Kuśnierczyk P. HLA-C C1C2 heterozygosity may protect women bearing the killer immunoglobulin-like receptor AA genotype from spontaneous abortion. *J Reprod Immunol*. 2011;88(1):32-7.
  22. Faridi RM, Agrawal S. Killer immunoglobulin-like receptors (KIRs) and HLA-C allorecognition patterns implicative of dominant activation of natural killer cells contribute to recurrent miscarriages. *Hum Reprod*. 2011;26(2):491-7.