Int J Clin Exp Pathol 2015;8(11):14535-14541
www.ijcep.com /ISSN:1936-2625/IJCEP0016885

Original Article

KIR2DL4 expression rather than its single nucleotide polymorphisms correlates with pre-eclampsia

Dandan Wang, Yuan Tian, Yanhui Zhao, Lianlian Liu, Xianying Liu, Fuju Wu

Department of Obstetrics and Gynecology, The Second Hospital of Jilin University, Changchun 130041, China

Received September 25, 2015; Accepted October 27, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Objective: To evaluate the single nucleotide polymorphisms and expression of KIR2DL4 (killer cell immunoglobulin-like receptors) gene in pre-eclampsia patients. Methods: KIR2DL4 gene polymorphisms were detected in 100 patients with pre-eclampsia and 100 healthy pregnant women, respectively, by using PCR-SS. Then, the expression of KIR2DL4 was measured in 5 cases of placentas tissues with pre-eclampsia and normal pregnancies by using qRT-PCR. Results: Compared with healthy controls, 16 loci of single nucleotide polymorphisms (SNP) were identified in pre-eclampsia patients, including 7 new polymorphisms loci. But, no significant difference was found in genotype distributions and allele frequencies in pre-eclampsia and controls ($P>0.05$). However, qRT-PCR results showed that KIR2DL4 mRNA in placenta tissues with pre-eclampsia was significantly lower than those with normal pregnancy, and the difference was statistically significant. Conclusion: Decreased level of KIR2DL4 rather than its SNP is correlated with the susceptibility of pre-eclampsia.

Keywords: KIR2DL4, single nucleotide polymorphisms, pre-eclampsia, susceptibility

Introduction

Pre-eclampsia (PE) is a severe immune-related disorder, which is characterized by the de novo onset of hypertension and proteinuria [1, 2]. It is reported that the incidence of pre-eclampsia ranges from 5% to 10% in the world [3, 4]. Also, this condition is the main cause of perinatal mortality and morbidity [5, 6]. However, the pathogenesis of PE remains unclear.

KIR2DL4 is a member of killer cell immunoglobulin-like receptors (KIR) family, which is characterized by both inhibitory and activating KIR [7]. KIR2DL4 can serve as a cell surface receptor in peripheral blood NK cells as well as uterine and decidual NK cells [8-10]. Recently, KIR2DL4 polymorphism was found in the Australian population [11] and correlated with risk for PE in multigravid pregnancies [12]. Here, we detected KIR2DL4 gene polymorphisms in 100 patients with pre-eclampsia and 100 healthy pregnant women from China. Moreover, the expression of KIR2DL4 was measured in 5 cases of placentas tissues with pre-eclampsia and normal pregnancies.

Materials and methods

Patients

A total of 100 preeclamptic and 100 healthy pregnant Chinese women between November 2012 and December 2013 were enrolled in this study. All women were from the Second Hospital of Jilin University, Changchun, China. Preeclamptic women were aged for 24-34 years and had 30-40 weeks' pregnant. Healthy pregnant woman were aged for 24-34 years and had 30-40 weeks' pregnant. The sample collection, selection criteria for cases and controls, as well as DNA isolation procedures have been described previously. All patients agreed with the informed consent and permitted to use samples for research. This study was approved by Ethics Committee of the Second Hospital of Jilin University, Changchun, China.

Genomic DNA extraction and genotyping

Genomic DNA was isolated from peripheral blood (2 ml) by TIANamp Blood DNA Kit (Generay Biotechnology, Shanghai, China) following the manufacturer's instructions. Briefly, blood sam-

Table 1. The SNP loci of KIR2DL4 gene

SNP number	region	location	nucleotide	*P* value
1	intron1	242	C>G	0.6152
2 (rs604076)	intron1	261	A>C	0.5698
3	intron2	375	G>A	0.7241
4 (rs2295803)	intron2	502	C>T	0.6594
5 (rs598452)	intron2	551	T>C	0.9646
6 (rs618835)	extron3	1264	A>G	0.3494
7	Intron3	1541	C>T	0.9345
8 (rs2075769)	*extron5*	*2380*	*C>T*	*0.0270*
9 (rs1051454)	extron5	2397	A>G	0.9135
10 (rs1051456)	extron5	2610	C>G	0.4419
11 (rs35837163)	intron5	5141	T>G	0.6675
12	intron5	5180	OO>AA	0.4066
13 (rs687423)	intron5	5220	A>G	0.7320
14	intron6	9417	C>T	0.1246
15	*intron6*	*9509*	*T>C*	*0.0046*
16	extron7	9578	C>T	0.1246
17 (rs604076)	intron8	10184/5	CT>AG	0.7893
18 (rs1051457)	extron9	10399	G>A	0.7322

Table 2. Distribution of alleles and genotypes in KIR2DL4 polymorphisms loci

Location	Alleles	Frequency		*P* value	Genotype	Frequency		*P* value
		PE	Control			PE	Control	
242	C	0.458	0.49	0.744	C/C	0.25	0.257	0.905
	G	0.542	0.51		C/G	0.417	0.457	
					G/G	0.333	0.286	
261	A	0.81	0.79	0.772	A/A	0.649	0.611	0.945
	C	0.19	0.21		A/C	0.324	0.361	
					C/C	0.027	0.028	
375	G	0.959	0.944	0.671	G/G	0.919	0.889	0.663
	A	0.041	0.056		G/A	0.081	0.111	
					A/A	0.000	0.000	
502	C	0.923	0.932	0.824	C/C	0.846	0.865	0.817
	T	0.077	0.068		C/T	0.154	0.135	
					T/T	0.000	0.000	
551	T	0.397	0.375	0.778	T/T	0.154	0.139	0.960
	C	0.603	0.625		T/C	0.487	0.472	
					C/C	0.359	0.389	
1264	A	0.675	0.671	0.963	A/A	0.475	0.486	0.957
	G	0.325	0.329		A/G	0.4	0.371	
					G/G	0.125	0.143	
1541	C	1	0.987	0.303	C/C	1	0.974	0.302
	T	0	0.013		C/T	0	0.026	
					T/T	0	0	
2397	A	0.539	0.566	0.744	A/A	0.289	0.316	0.946
	G	0.461	0.434		A/G	0.5	0.5	
					G/G	0.211	0.184*	

ples was mixed with Boiled RNase A, Proteinase K and TBM Solution at 55°C for 10 min. The supernatant was collected and transferred to GenClean Column. Then, DNA fluid was obtained by centrifugation by using wash solution and Elution Buffer. DNA concentration was measured and stored at -20°C until analysis. Then, PCR-SSP was performed by following the manufacturer's instructions. Briefly, each 50 μl PCR reaction mixture contained 5 μl LA PCR Buffer, 4 μl $MgCl_2$, 10 μl deoxynucleotide triphosphate (dNTP), 8 μl pairs of forward and reverse primers (**Table 1**), 5 μl Templete, 1 μl LA Taq DNA Polymerase, 1 μl dimethylsulfoxide (DMSO) and 16 μl ddH_2O. Polymerase chain reaction conditions were as follows: a 5-minute initial denaturation at 94°C, followed by 5 cycles of 94°C for 30 seconds, 61°C for 45 seconds, 68°C for 6 minutes, and ending with a final extension step at 68°C for 10 minutes. A total of 3 μl PCR product was mixed with 1 μl Loading Buffer and analyzed by electrophoresis through a 0.8% agarose gel for 1 hour. In order to detect the SNP in intron and exon of KIR2DL4, we performed DNA sequencing analysis in exon 2, 3, 5, 6, 7, 8 and 9 by designing the amplification sequences of KIR2DL4 gene (**Table 2**).

2610	C	0.461	0.434	0.744	C/C	0.237	0.158	0.587
	G	0.539	0.566		C/G	0.447	0.553	
					G/G	0.316	0.289	
5141	T	0.5	0.441	0.506	T/T	0.267	0.176	0.684
	G	0.5	0.559		T/G	0.467	0.529	
					G/G	0.267	0.294	
5180*	OO	0.516	0.426	0.306	OO/OO	0.258	0.147	0.511
	AA	0.484	0.574		OO/AA	0.516	0.559	
					AA/AA	0.226	0.294	
5220	A	0.962	0.947	0.673	A/A	0.923	0.895	0.665
	G	0.038	0.053		A/G	0.077	0.105	
					G/G	0	0	
9417	C	0.763	0.851	0.164	C/C	0.65	0.757	0.474
	T	0.238	0.149		C/T	0.225	0.189	
					T/T	0.125	0.054	
9578	C	0.763	0.851	0.164	C/C	0.65	0.757	0.474
	T	0.238	0.149		C/T	0.225	0.189	
					T/T	0.125	0.054	
10184/5	CT	0.775	0.855	0.198	CT/CT	0.650	0.737	0.391
	GA	0.225	0.145		CT/GA	0.250	0.237	
					GA/GA	0.100	0.026	
10399	G	0.500	0.419	0.316	G/G	0.231	0.189	0.510
	A	0.500	0.581		G/A	0.538	0.459	
					A/A	0.231	0.351	

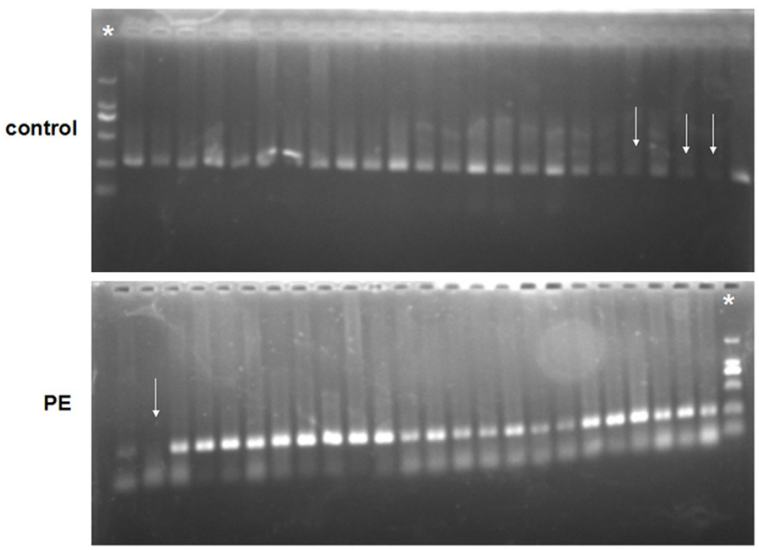

Figure 1. Electrophoretogram of polymerase chain reaction for KIR2DL4. *: molecular weight marker, white arrow: negative for KIR2DL4.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was abstracted from 5 cases of placentas tissues with pre-eclampsia and 5 cases of normal pregnancies tissues by Trizol reagent (TAKARA, Japanese) and reversely transcribed into cDNA by using Reverse Transcription System (TAKARA, Japanese). The PCR analysis of TRAP1 gene expression was performed by using the SYBR Green RT-PCR Kit (TAKARA, Japanese). The primer sequences of KIR2DL4 were (F) 5' CTTCCCTTCTTTCTCCTTCATCG 3' and (R): 5'-CACATCAAACATGGACGGTTT-3'. GAPDH was used as internal control and its primer sequences of were (F) 5'-AGGTCGGTGTGAACGGATTTG-3' and (R) 5'-TGTAGACCATGTAGTTGAGGTCA-3'. All primers were purified and synthesized by the Huada Company (HuaDa, Shenzhen, China). Real-time PCR cycle conditions were one cycles of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 (SPSS. Inc., Chicago, IL, USA). The Hardye Weinberg equilibrium of KIR2DL4 gene allele frequencies was tested. The chi-square test was used to compare allele and genotype frequencies between pre-eclamptic and healthy controls. The difference of KIR2DL4 mRNA level in pre-eclamptic patients and healthy controls was assessed by independent samples *t* test. Statistical significance was set at $P<0.05$.

Results

The SNP of KIR2DL4 was systematically examined by amplification and sequencing of genomic DNA. The results revealed that 3 cases in the healthy controls and 1 cases in preeclamptic patients were negative for the framework

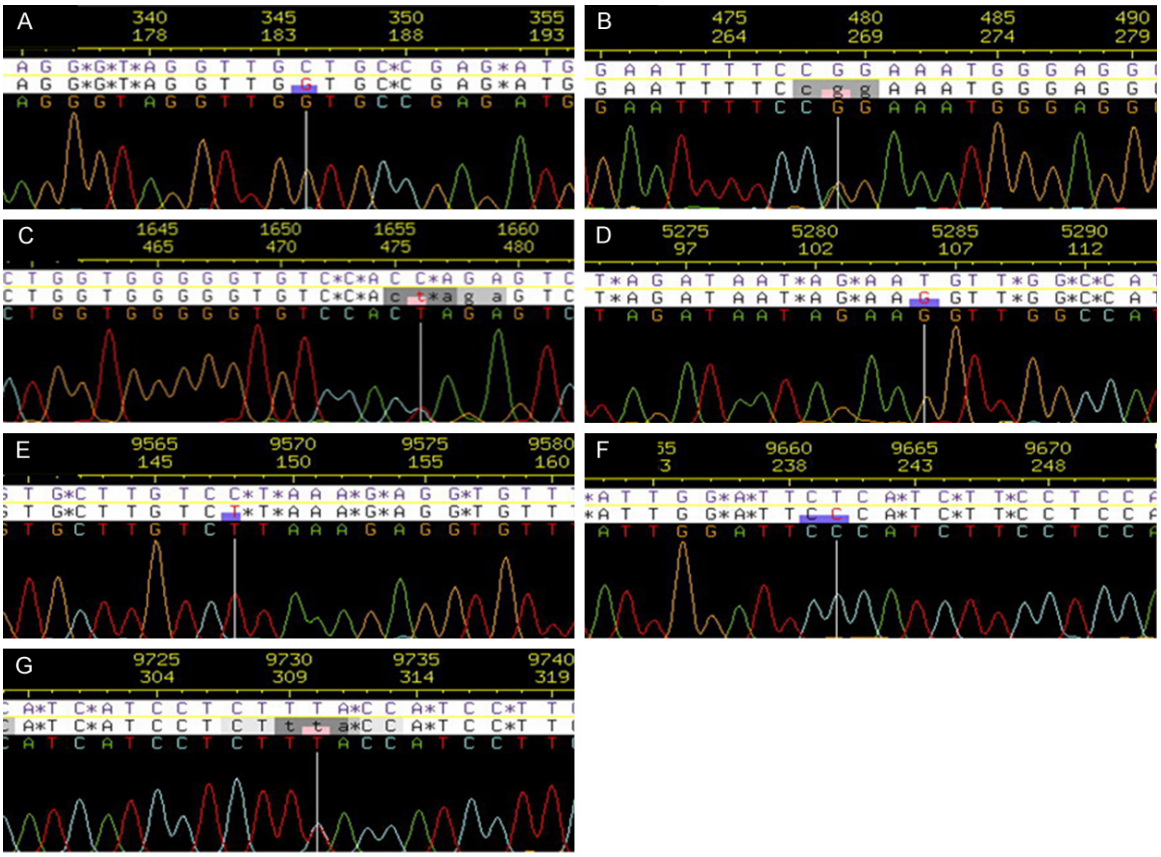

Figure 2. Seven novel SNP loci of KIR2DL4 were confirmed by DNA sequencing analysis. A. Sequencing peak pattern of SNP in KIR2DL4 242/346 loci. B. Sequencing peak pattern of SNP in KIR2DL4 375/479 loci. C. Sequencing peak pattern of SNP in KIR2DL4 1541/1656 loci. D. Sequencing peak pattern of SNP in KIR2DL4 5141/5284 loci. E. Sequencing peak pattern of SNP in KIR2DL4 9417/9568 loci. F. Sequencing peak pattern of SNP in KIR2DL4 9509/9662 loci. G. Sequencing peak pattern of SNP in KIR2DL4 9578/9731 loci.

KIR2DL4 gene, and all other samples from both controls and patient groups were positive (**Figure 1**). However, the difference between controls and patient groups was not significant (P>0.05). DNA sequencing analysis showed that there were 18 SNP loci in the exon-intron junction of KIR2DL4 gene in preeclamptic patients and healthy controls, including 13 conversions and 4 transversion and 1 insertion mutation (**Table 1**). Meanwhile, there were 7 novel SNP loci that had not been previously described in NCBI's dbSNP (**Figure 2**). In 18 SNPs loci, six (four non-synonymous substitutions and two synonymous substitutions) were located in coding regions and the others were located in the exon-intron junction. These SNP loci except 2380 and 9509 were accorded with Hardye Weinberg equilibrium (P>0.05).

Then, we analyzed the distribution of alleles and genotype in the 16 polymorphic loci of KIR2DL4 gene (excluding 2380 and 9509) in the preeclampsia and normal pregnancy group. As shown in **Table 2**, no significant difference was found in alleles and genotype distribution between the two groups (P>0.05).

In addition, to evaluate whether KIR2DL4 expression is correlated with pre-elampsia toxemia, we detected the KIR2DL4 level in 5 cases of placentas tissues from preeclamptic patients and healthy controls by qRT-PCR. The quality of RNA and the specificity of primer sequence were confirmed to be reliable (**Figure 3**). Then, we found that KIR2DL4 level in preeclamptic patients was significantly decreased compared with healthy controls (P<0.05, **Figure 4**).

Discussion

NK cells are important immune cells to defense against tumor cells or virus-infected cells and

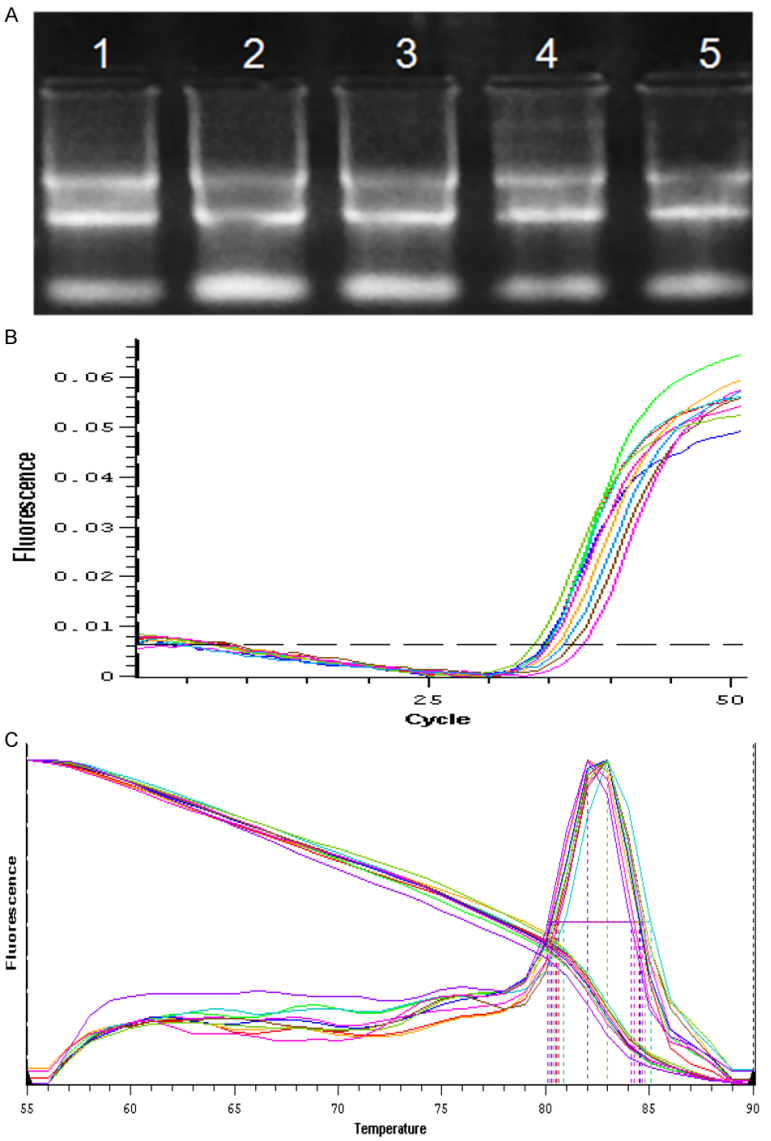

Figure 3. The quality of RNA and the specificity of primer sequence were evaluated. A. Electrophoretogram of RNA between PE patients (lane 1-3) and healthy controls (lane 4-5). B. Amplification Plot of KIR2DL4 gene. C. Solubility curve of KIR2DL4 gene.

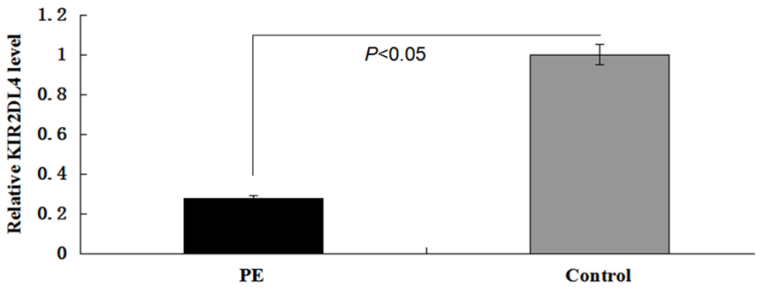

Figure 4. KIR2DL4 mRNA level in preeclamptic patients and healthy controls was detected by QRT-PCR.

to soluble mediators, and are prevalent in uterine leukocyte population during the early stages of pregnancy, which play key roles in uterine tissue remodeling [13]. During the stages of successful pregnancy, NK cells located in uterine secrete inflammatory and angiogenic factors to promote the growth, differentiation, and migration of trophoblast cells and the remodeling of spiral artery [13]. Recent studies suggest that KIR2DL4 may play a role in this process.

KIR2DL4 is a member of killer cell immunoglobulin-like receptors (KIRs) family. Based on the number of extracellular domains, the length of cytoplasmic tail and the sequence similarity, KIR proteins are classified into inhibitory and activating KIR [14]. However, KIR2DL4 has distinct expression, cellular localization, structure and function among KIR members. Studies have indicated that the KIR2DL4 is expressed by all NK cells and some T cells at the transcriptional level, while its surface expression is variable [15, 16]. Compare with other KIRs, KIR2DL4 has a D1 and D2 domain structure, and comprises multiple exons. Furthermore, it is unique that KIR2DL4 only possesses a single immune tyrosine-based inhibitory motif (ITIM) and an arginine residue in the transmembrane region. Functionally, KIR2DL4 can regulate the cytokine secretion and cytotoxicity. In addition, KIR2DL4 is characterized by its high degree of conservation and low polymorphism

among the highly variable KIR family members [13].

Several studies have reported that KIR2DL4 is a specific receptor for HLA-G [16, 17]. HLA-G, a non-classical MHC class I molecule, is expressed only by fetal-derived trophoblast cells in pregnant women and can create a barrier to prevent the fetus from maternal NK cell attack [10]. The reduced levels of HLA-G in pregnant women have been reported to be associated with pre-eclampsia and recurrent spontaneous abortion [18-20]. Thus, it seems to imply that the interaction of KIR2DL4 with HLA-G may play an important role in the maintenance of pregnancy. Nonetheless, Nowak et al. reported that a Polish woman who is lacking KIR2DL4 gene had a normal pregnancy and delivered a child [21]. Moreover, the relationship between KIR2DL4 and pre-eclampsia is rare to report. Witt et al. reported that alleles of the KIR2DL4 were significantly related to pre-eclampsia in the Australian population [11]. While Tan et al. reported that the possible gene-gene interaction of KIR2DL4 with HLA-G*0106 is correlated with the risk of PE in Malaysia population [12].

In present study, we detected the SNP of KIR2DL4 gene in preeclamptic patients and healthy controls from China. The results revealed that 97% cases of healthy controls and 99% cases of preeclamptic patients were positive for KIR2DL4 gene, indicating that KIR2DL4 gene may be important for both healthy population and preeclamptic patients. In addition, the results demonstrated that there were 18 SNP loci in the exon-intron junction of KIR2DL4 gene, including 13 conversions and 4 transversion and 1 insertion mutation. Meanwhile, there were 7 novel SNP loci that have not been previously described in NCBI's dbSNP. These SNP loci except 2380 and 9509 were accorded with Hardye Weinberg equilibrium. However, no significant difference was recorded in alleles and genotype distribution between preeclamptic patients and healthy controls. These data were consistent with the studies reported by Witt [11] and Tan [12], indicating that KIR2DL4 SNP was not significantly correlated with the susceptibility of pre-eclampsia. In addition, Zhu et al. reported that Chinese Han population has distinct allele frequencies of KIR2DL4 in comparison to some other populations [22]. Then, to further evaluate whether KIR2DL4 expression is correlated with pre-elampsia toxemia, we detected the KIR2DL4 mRNA level in preeclamptic patients and healthy controls by qRT-PCR. The results revealed that KIR2DL4 mRNA level in pre-eclamptic patients was significantly decreased compared with healthy controls, suggesting that KIR2DL4 expression was associated with pre-eclampsia. It is well known that HLA-G is the ligand of KIR2DL4 and associated with pre-eclampsia [19, 20]. Thus, we considered that the decrease of KIR2DL4 in preeclamptic patients might be connected with the reduced level of HLA-G. Of course, further investigation is needed.

In conclusion, this study demonstrates that the SNP loci of KIR2DL4 gene is found in pre-eclamptic patients. While the SNP of KIR2DL4 gene is not significantly correlated with the susceptibility of pre-eclampsia. However, the decreased level of KIR2DL4 may be connected with the occurrence of pre-eclampsia.

Acknowledgements

This study was supported by grants from the Jilin Provincial Science & Technology Department, Jilin Province, China, 20130206-057SF.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Fuju Wu, Department of Obstetrics and Gynecology, The Second Hospital of Jilin University, 218 Ziqiang Street, Nanguan District, Changchun 130041, China. Tel: +86-431-88796162; Fax: +86-431-88796162; E-mail: wufuju1212@sina.com

References

[1] Aquino LO, Leite HV, Cabral AC, Brandão AH. Doppler flowmetry of ophthalmic arteries for prediction of pre-eclampsia. Rev Assoc Med Bras 2014; 60: 538-541.

[2] Hu W, Wang H, Huang H. Analysis of gene expression and preliminary study of methylation about 11beta-HSD2 gene in placentas of Chinese pre-eclampsia patients of Han ethnicity. J Obstet Gynaecol Res 2015; 41: 343-349.

[3] Olatunji RB, Adekanmi AJ, Obajimi MO, Roberts OA, Ojo TO. Maternal ophthalmic artery Doppler velocimetry in pre-eclampsia in Southwestern Nigeria. Int J Womens Health 2015; 7: 723-734.

[4] Ge J, Wang J, Zhang F, Diao B, Song ZF, Shan LL, Wang W, Cao HJ, Li XQ. Correlation between MTHFR gene methylation and pre-eclampsia, and its clinical significance. Genet Mol Res 2015; 14: 8021-8028.
[5] Bergink V, Laursen TM, Johannsen BM, Kushner SA, Meltzer-Brody S, Munk-Olsen T. Pre-eclampsia and first-onset postpartum psychiatric episodes: a Danish population-based cohort study. Psychol Med 2015; 45: 3481-3489.
[6] Cormick G, Zhang NN, Andrade SP, Quiroga MJ, Di Marco I, Porta A, Althabe F, Belizán JM. Gaps between calcium recommendations to prevent pre-eclampsia and current intakes in one hospital in Argentina. BMC Res Notes 2014; 7: 920.
[7] Rajagopalan S, Long EO. KIR2DL4 (CD158d): an activation receptor for HLA-G. Front Immunol 2012; 3: 258.
[8] Goodridge JP, Lathbury LJ, John E, Charles AK, Christiansen FT, Witt CS. The genotype of the NK receptor, KIR2DL4, influences IFNgamma secretion by decidual natural killer cells. Mol Hum Reprod 2009; 15: 489-497.
[9] Hromadnikova I, Pirkova P, Sedlackova L. Influence of in vitro IL-2 or IL-15 alone or in combination with Hsp-70-derived 14-mer peptide (TKD) on the expression of NK cell activatory and inhibitory receptors. Mediators Inflamm 2013; 2013: 405295.
[10] Yan WH, Lin A, Chen BG, Zhou MY, Dai MZ, Chen XJ, Gan LH, Zhu M, Shi WW, Li BL. Possible roles of KIR2DL4 expression on uNK cells in human pregnancy. Am J Reprod Immunol 2007; 57: 233-242.
[11] Witt CS, Whiteway JM, Warren HS, Barden A, Rogers M, Martin A, Beilin L, Christiansen FT. Alleles of the KIR2DL4 receptor and their lack of association with pre-eclampsia. Eur J Immunol 2002; 32: 18-29.
[12] Tan CY, Chong YS, Loganath A, Chan YH, Ravichandran J, Lee CG, Chong SS. Possible gene-gene interaction of KIR2DL4 with its cognate ligand HLA-G in modulating risk for pre-eclampsia. Reprod Sci 2009; 16: 1135-1143.
[13] Vacca P, Moretta L, Moretta A, Mingari MC. Origin, phenotype and function of human natural killer cells in pregnancy. Trends Immunol 2011; 32: 517-523.
[14] Schellekens J, Tilanus MG, Rozemuller EH. The elucidation of KIR2DL4 gene polymorphism. Mol Immunol 2008; 45: 1900-1906.
[15] Rajagopalan S, Long EO. KIR2DL4 (CD158d): An activation receptor for HLA-G. Front Immunol 2012; 3: 258.
[16] Li G, Weyand CM, Goronzy JJ. Epigenetic mechanisms of age-dependent KIR2DL4 expression in T cells. J Leukoc Biol 2008; 84: 824-834.
[17] Gómez-Lozano N, Trompeter HI, de Pablo R, Estefanía E, Uhrberg M, Vilches C. Epigenetic silencing of potentially functional KIR2DL5 alleles: Implications for the acquisition of KIR repertoires by NK cells. Eur J Immunol 2007; 37: 1954-1965.
[18] Gómez-Lozano N, de Pablo R, Puente S, Vilches C. Recognition of HLA-G by the NK cell receptor KIR2DL4 is not essential for human reproduction. Eur J Immunol 2003; 33: 639-644.
[19] LeMaoult J, Zafaranloo K, Le Danff C, Carosella ED. HLA-G up-regulates ILT2, ILT3, ILT4, and KIR2DL4 in antigen presenting cells, NK cells, and T cells. FASEB J 2005; 19: 662-664.
[20] Rizzo R, Vercammen M, van de Velde H, Horn PA, Rebmann V. The importance of HLA-G expression in embryos, trophoblast cells, and embryonic stem cells. Cell Mol Life Sci 2011; 68: 341-352.
[21] Yie SM, Li LH, Li YM, Librach C. HLA-G protein concentrations in maternal serum and placenta tissue are decreased in preeclampsia. Fertil Steril 2004; 83:30-36.
[22] Steinborn A, Varkonyi T, Scharf A, Bahlmann F, Klee A, Sohn C. Early detection of decreased soluble HLA-G levels in the maternal circulation predicts the occurrence of preeclampsia and intrauterine growth retardation during further course of pregnancy. Am J Reprod Immunol 2007; 57: 277-286.
[23] Nowak I, Majorczyk E, Płoski R, Senitzer D, Sun JY, Kuśnierczyk P. Lack of KIR2DL4 gene in a fertile Caucasian woman. Tissue Antigens 2011; 78: 115-119.
[24] Zhu FM, Jiang K, Lv QF, He J, Yan LX. Investigation of killer cell immunoglobulin-like receptor KIR2DL4 diversity by sequence-based typing in Chinese population. Tissue Antigens 2006; 67: 214-221.