Original article

Human neutrophil antigen-3 allele mismatching among kidney transplant patients with rejection

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Abstract:

Introduction: *To date, the study of HNA-3 genotype mismatching in kidney-transplant patients with antibody-mediated rejection (AMR) has not been reported.* ***Objective:*** *To determine the extent of HNA-3 genotype mismatching and to estimate the risk of HNA-3 alloimmunization and also detect anti-HNA-3 antibodies among kidney-transplant patients with AMR.* ***Materials and Methods:*** *Ninety pairs of recipients and the corresponding donors were genotyped for the HNA-3 system and an HNA-3a variant (C451T) by polymerase chain reaction using sequence-specific primers (PCR-SSP). HNA-3 antibodies were investigated using the granulocyte agglutination test (GAT) and the LabScreen Multi HNA kit.* ***Results:*** *Patients with AMR had frequencies of HNA-3a/3a, HNA-3a/3b and HNA-3b/3b of 0.567, 0.355 and 0.078, respectively, while the frequencies in the corresponding donors were 0.556, 0.433 and 0.011, respectively. The risk of alloimmunization for HNA-3a was 0.072. HNA-3a incompatibility was found in 21% and HNA-3b in 79%. No AMR patients exhibited HNA-3a and HNA-3b antibodies. However, patients with AMR due to HLA-class I and/or class II antibodies were found.* ***Conclusion:*** *Our data provide evidence that the chance of HNA-3 incompatibility in kidney-transplant recipients is high (36.7% in this study) despite which, HNA-3a antibodies were not detected. Taken together, these findings indicate that HNA-3a antibodies are less important for the pathomechanism of kidney transplant rejection.*

Keywords : ● *Human neutrophil antigen* ● *Graft rejection* ● *Antibody mediated rejection* ● *Kidney transplantation*

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นิพนธ์ต้นฉบับ

Human neutrophil antigen-3 allele mismatching ในผู้ป่วยปลูกถ่ายไตที่มีการปฏิเสธอวัยวะ

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[1]นักศึกษาปริญญาโท สาขาเทคนิคการแพทย์ คณะเทคนิคการแพทย์ [2]ศูนย์วิจัยและพัฒนาการตรวจวินิจฉัยทางห้องปฏิบัติการทางการแพทย์ คณะเทคนิคการแพทย์ [3]ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ [4]กลุ่มวิชาภูมิคุ้มกันวิทยาคลินิกและวิทยาศาสตร์การบริการโลหิต คณะเทคนิคการแพทย์ [5]คลังเลือดกลาง คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

บทคัดย่อ

บทนำ *ปัจจุบันยังไม่มีการรายงานการศึกษาการเกิดความไม่เข้ากันของจีโนไทป์ HNA-3 ในผู้ป่วยปลูกถ่ายไตที่เกิดการปฏิเสธไตจากแอนติบอดี* ***วัตถุประสงค์*** *เพื่อศึกษาการเกิดความไม่เข้ากันของจีโนไทป์ HNA-3 และประเมินโอกาสเสี่ยงการสร้างแอนติบอดี และตรวจหาแอนติบอดีต่อ HNA-3 ในผู้ป่วยปลูกถ่ายไตที่ปฏิเสธไตจากแอนติบอดี* ***วัสดุและวิธีการ*** *ผู้ป่วยและคู่ผู้บริจาคจำนวน 90 คู่ ถูกตรวจหาจีโนไทป์ของ HNA-3 และ HNA-3a variant (C451T) ด้วยเทคนิคพีซีอาร์เอสเอสพี และตรวจหาแอนติบอดี HNA-3 ด้วยเทคนิค granulocyte agglutination test (GAT) และ LabScreen Multi HNA kit* ***ผลการศึกษา*** *ผู้ป่วยที่มีการปฏิเสธไตจากแอนติบอดีมีความถี่จีโนไทป์ HNA-3a/3a HNA-3a/3b และ HNA-3b/3b เท่ากับ 0.567 0.355 และ 0.078 ตามลำดับ ส่วนผู้บริจาคมีความถี่เท่ากับ 0.556 0.433 และ 0.011 ตามลำดับ พบโอกาสเสี่ยงการสร้างแอนติบอดี HNA-3a เท่ากับ 0.072 โดยพบการเกิดความไม่เข้ากันของแอนติเจน HNA-3a เท่ากับ ร้อยละ 21 และแอนติเจน HNA-3b ร้อยละ 79 และไม่พบผู้ป่วยที่สร้างแอนติบอดี HNA-3a และ HNA-3b อย่างไรก็ตาม พบว่าผู้ป่วยมีการปฏิเสธไตจากแอนติบอดีต่อ HLA-class I และหรือ HLA-class II* ***สรุป*** *การศึกษานี้แสดงให้เห็นว่า โอกาสเกิดความไม่เข้ากันของแอนติเจน HNA-3 ในผู้ป่วยปลูกถ่ายไตสูงถึง ร้อยละ 36.7 แม้ว่าจะตรวจไม่พบแอนติบอดี HNA-3a จากข้อมูลทั้งหมด บ่งชี้ให้เห็นว่าแอนติบอดีต่อ HNA-3a มีความสำคัญน้อยกับกลไกการเกิดการปฏิเสธไต*

คำสำคัญ : ● *แอนติเจนเม็ดเลือดขาว* ● *การปฏิเสธอวัยวะ* ● *การปฏิเสธอวัยวะจากแอนติบอดี* ● *การปลูกถ่ายไต*

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต. 2564;31:235-42.

Introduction

Human neutrophil antigen 3 (HNA-3) is polymorphic and resides on the choline-transporter-like protein 2 (CTL-2) encoded by a gene (*solute carrier protein 44A2*, *SLC44A2*) located on chromosome 19p13.2.[1] CTL-2 is a 68-72 kDa glycoprotein composed of 10 transmembrane domains, 5 extracellular peptide loops and 6 intracellular regions. CTL-2 is not only expressed on neutrophils but also lymphocytes, platelets, and many organ tissues including kidney and pulmonary endothelium cells.[2,3] HNA-3a and HNA-3b alleles differ only at a single amino-acid position: Arg 152 (HNA-3a, *SLC44A2*01*) and Gln152 (HNA-3b, *SLC44A2*02*).[1,4] An additional SNP (451C>T; Leu151Phe; rs147820753), called HNA-3a variant, impairs HNA-3a antibody binding and can impact correct genotyping of HNA-3a if the specific primer is constructed with this alteration.[5] This indicates a need to also genotype for the HNA-3a variant.

HNA-3a antibodies are implicated in severe and fatal transfusion-related acute lung injury (TRALI).[6,7] These antibodies also have a role in many diseases such as allo- and auto-immune neutropenia, febrile-nonhemolytic transfusion reaction and autoimmune hearing loss.[8] HNA-3a antigen is a genetic risk factor for recurrent venous thromboembolism, as revealed by genome-wide association studies.[9]

Population studies have shown that frequencies of HNA-3 alleles vary among populations and countries. About 5-6% of American and European individuals are homozygous for HNA-3b. In Asia, 5-6% of Japanese, 22% of the Yi, and 12% of the Tibetan population in China and around 5% of Thais are homozygous for the HNA-3b allele.[10,11] Being homozygous for HNA-3b is a risk factor for alloimmunization to HNA-3a. However, anti-HNA-3a is much less frequently reported in Asian than in European populations.[12,13]

Antibodies that are specific to organ-donor human leukocyte antigen (HLA)-specific antibodies have been involved in the majority of cases of antibody-mediated rejection (AMR) in kidney transplantation when cross-matches were positive T-cell and B- cell. AMR can occur even in the apparent absence of HLA donor-specific antibodies (HLA-DSA), indicating that polymorphic non-HLA antibodies can also cause poor transplant outcomes.[14,15] Clinically, vascular endothelium is the first barrier between the recipient's immune system and the allograft in vascularized organ transplantation such as kidney transplantation.[16] Thus, it is commonly thought that endothelial cells of kidney vessels are the main targets of kidney rejection. Until today, only one study has reported that AMR can be driven by anti-HNA-3a antibodies in kidney transplant recipients.[17] Those observations may bring to the forefront the discussion about the clinical relevance of HNA-3a antibodies in transplantation and the need for HNA-3 genotyping prior to transplantation, something which is not routinely done.

This study aimed to determine the extent of HNA-3 genotype mismatching and to estimate the risk of HNA-3 alloimmunization and also detect anti-HNA-3 antibodies among Thai kidney-transplant patients with AMR.

Materials and Methods

Sample collection

DNA samples of recipients diagnosed as experiencing AMR according to the Banff 2017 classification[18] and their corresponding donors were obtained from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. DNA from 90 donor/recipient pairs was genotyped for the HNA-3 system, and post-transplant serum samples were tested for the presence of HNA-3 antibodies to identify cases of HNA-3 incompatibility and to explore possible association of such antibodies with rejection. The Ethics Committee of Khon Kaen University approved this study (HE621078).

HNA-3 genotyping

Genotyping of HNA-3 of the recipients and the corresponding donors was performed using polymerase chain reaction with sequence-specific primers (PCR-SSP), as described previously.[11,19] Briefly, PCR reactions were performed in a total of 13 μL of 1×PCR buffer and 0.50 μM of the specific primers. For the internal control, human growth hormone (0.1 μM) was used. The

amplification process consisted of an initial denaturation for 2 min at 96°C, then 5 cycles of 30 s at 96°C, 60 s at 68°C, and 40 s at 72°C, then 21 cycles of 30 s at 96°C, 60 s at 57°C, 40 s at 72°C, then 4 cycles of 30 s at 96°C, 1.15 min at 55°C, 2 min at 72°C, followed by 72°C for 10 min. The sample was then kept at 4°C in the thermocycler (Applied Biosystems Veriti ™ Thermal Cycler, Life Technologies, Foster City, CA). The amplified PCR products were evaluated by electrophoresis in 1.5% agarose gels in 0.5× Tris borate ethylenediaminetetraacetate (TBE) buffer stained with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA). The PCR bands were visualized under UV light using the gel documentation system (UVItec, Cambridge, UK).

HNA-3a variant typing (C451T polymorphism)

Genotyping of HNA-3a variant C451T was also performed using the PCR-SSP method described previously.[11] To validate the reliability of the genotyping, we used a positive control for the C451T polymorphism from the previous study.[11]

Defining HNA-3 incompatibility

We classified HNA-3 incompatibility into two types. If the recipient is HNA-3b homozygous and the corresponding donor is HNA-3a homozygous or heterozygous, it is called HNA-3a incompatibility. The reverse situation leads to HNA-3b incompatibility.

HNA-3 antibody testing

Granulocyte agglutination test (GAT)

Detection of HNA-3 antibodies was performed using the GAT as previously described.[20] Briefly, the concentration of cells in a suspension of freshly isolated neutrophils (dextran sedimentation method) was adjusted to 5 x 10^3 neutrophils/μL. Patient sera were incubated with panels of neutrophils prepared from four neutrophil donors with known genotypes (two with HNA-3a/3a and two with HNA-3b/3b) on Terasaki plates for 120 minutes at 37°C. All samples were tested in duplicate. Anti-HNA-3a positive serum (supplied by the Institute for Clinical Immunology and Transfusion Medicine, Giessen, Germany) was used as a control. The results were evaluated under an inverted microscope (OLYMPUS Optical, Japan) and considered positive if agglutination occurred.

LabScreen Multi-HNA kit

A multiplex assay, LabScreen Multi-HNA mixed kit (One Lambda, Inc.), was used to simultaneously detect the antibodies against purified peptides of both HLA class I and II and HNA. The protocols were performed according to the manufacturer's instruction and the Luminex flow analyzer software was used for the data acquisition and analyses. Recommended normalized background ratio (NBG) > 2.2 was used as the cut-off for antibody positivity.

Statistical analysis

Demographic data of recipients were tabulated and the comparison between groups and demographic variables was calculated using the Chi-square test. For the calculation of gene frequencies, the direct-counting method and Hardy-Weinberg equilibrium were assessed by the web-based calculator (http://wpcalc.com/en/equilibrium-hardy-weinberg/). The risk of alloimmunization against the HNA system was calculated using formulae as previously described.[19] *P*-values less than 0.05 were considered significant. All statistical analyses were performed using Graph Pad Prism version 8.0.0, Graph Pad Software, San Diego, California USA.

Results

Patient demographics

We studied 90 pairs of kidney transplant recipients and the corresponding donors. Recipients in the HNA-3 matched and HNA-3 mismatched groups had a mean age of 40.23 years and 40.19 years, respectively. Between these two groups, there were no significant differences in terms of sex, age, and type of donor (living or deceased) as shown in Table 1.

HNA-3 genotypes frequencies in the study

The genotype frequencies of HNA-3 in 90 renal-transplant recipients with AMR and the corresponding donors are shown in Table 2. Frequencies of genotypes HNA-3a/3a,

Table 1 Demographic details of the 90 kidney transplant recipients

Recipients' base-line demographic	HNA-3 compatible	HNA-3 incompatible	*p*-value
Number	57 (63%)	33 (37%)	
Recipients' sex			
Male n (%)	40 (70%)	16 (48%)	0.069
Female n (%)	17 (30%)	17 (52%)	
Recipients' age (Mean ± SD)	40.23 ± 12.19	40.19 ± 13.56	0.988
Donors and transplant characteristics			
Live donor transplant n (%)	6 (11%)	3 (10%)	0.827
Deceased donor transplant n (%)	51 (89%)	30 (90%)	

Table 2 Genotype and allele frequencies of HNA-3 in kidney transplant recipients and the corresponding donors

Genotype	Recipients (n = 90)				Donors (n = 90)			
	Observed frequency	Expected frequency	Allele frequency	HDW	Observed frequency	Expected frequency	Allele frequency	HDW
3a3a	0.567	0.554	a = 0.744	x^2 = 0.304	0.556	0.597	a = 0.772	x^2 = 4.078
3a3b	0.355	0.380	b = 0.256	*p* = 0.859	0.433	0.352	b = 0.228	*p* = 0.130
3b3b	0.078	0.066			0.011	0.052		

HDW = Hardy-Weinberg equilibrium

HNA-3a/3b and HNA-3b/3b among recipients were 0.567, 0.355, and 0.078, respectively. Corresponding figures for donors were 0.556, 0.433, and 0.011, respectively. These frequencies were consistent with Hardy-Weinberg equilibrium in both recipient and donor groups (p = 0.859 and p = 0.130, respectively). In detail, 7 of 90 (7.8%) recipients and only one (1.1%) of the corresponding donors were homozygous for HNA-3b. The allele frequencies of HNA-3a and HNA-3b were 0.744 and 0.256 in recipients and 0.772 and 0.228 in donors. In addition, to avoid misidentification of heterozygotes as HNA-3b/3b, SNP C451T was also assayed in all samples and no cases were detected.

Estimated risk of HNA-3a incompatibility and alloimmunization

The estimated risk of HNA-3a alloimmunization was 0.072. Thirty-three of the 90 pairs (36.7%) were mismatched for HNA-3. Seven out of 33 (21%) were mismatched for HNA-3a and the remainder were mismatched for HNA-3b, as shown in Table 3.

Table 3 Detailed description of HNA-3 incompatibility in kidney transplant rejection

HNA-3 incompatibility	Kidney allograft recipients
HNA-3a incompatibility	
Donor: 3aa or 3ab	
Recipients: 3bb	7 (21%)
HNA-3b incompatibility	
Donor: 3bb or 3ab	
Recipients: 3aa	26 (79%)

HNA-3 antibody testing

GAT was performed to detect anti-HNA-3 antibodies. The GAT was considered positive when the patient serum showed agglutination with two HNA-3a/a or HNA-3b/b donors. Agglutination was not seen in any of the sera from the 33 recipients mismatched for HNA-3 relative to their donors (data not shown). However, a reference serum known to have anti-HNA-3a antibodies showed agglutination with known HNA-3a/a neutrophils but not with HNA-3b/b neutrophils (Figure 1)

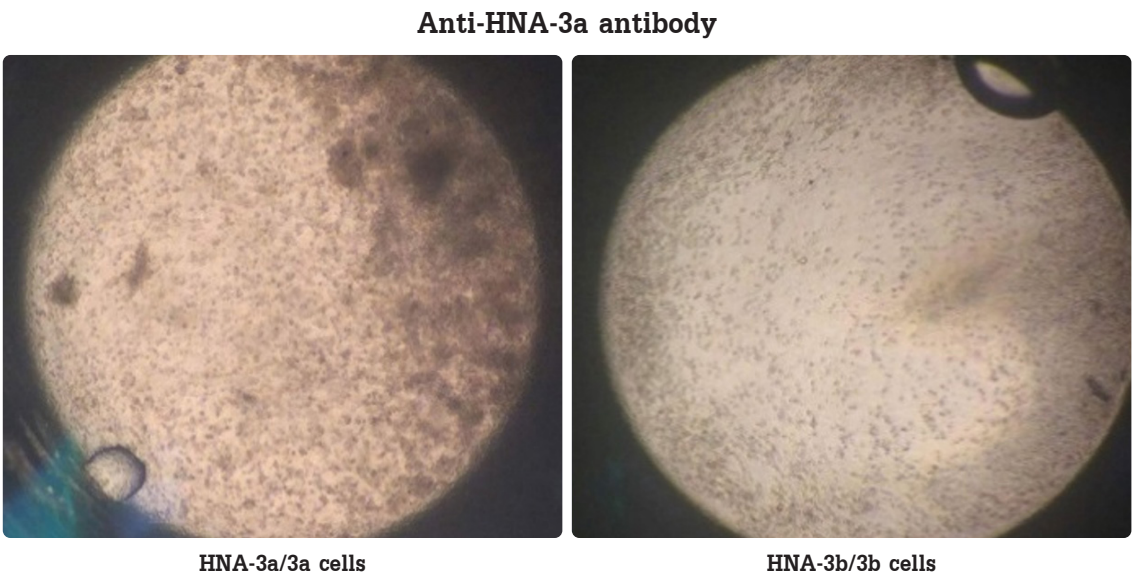

Figure 1 Granulocyte agglutination test: reaction patterns of a serum known to have anti-HNA-3a antibodies with HNA-3a/a and HNA-3b/b neutrophil suspensions

The LabScreen Multi HNA kit found no positive examples for anti-HNA-3a or HNA-3b antibodies. In contrast, the kit detected three cases with class I anti-HLA, six with class II anti-HLA, and five with class I and II HLA antibodies (data not shown).

Discussion

HNA-3a antibodies exhibit strong crosslinking activity with their cognate antigens on endothelial cells, causing endothelial barrier dysfunction, increased reactive oxygen species production (ROS), increased permeability, increased albumin influx pumping and subsequently the TRALI reaction.[6] However, no case of TRALI associated with anti-HNA-3b antibody has been reported, although one study found this antibody associated with neonatal alloimmune neutropenia (NAIN) in Brazil.[21] Besides TRALI and NAIN, a more recent study has reported that HNA-3a antibodies were implicated in a case of kidney rejection, and remains the only study to show an association between HNA-3a antibodies and transplantation.[17] Here, we report for the first time the risk of HNA-3 genotype mismatching and alloimmunization among Thai kidney-transplant recipients with AMR. This has also been the first investigation of HNA-3 antibodies in a genotype-mismatched group.

We found the frequency of HNA-3a/3a in Thai kidney-transplant patients to be much higher than that of HNA-3b/3b (0.567 and 0.078, respectively). No cases of the SNP C451T variation were observed, similar to a previous study.[11] Among our kidney-transplant patients with AMR, 36.7% were mismatched for HNA-3 genotype relative to their donors. The estimated risk of alloimmunization for HNA-3a was 0.072. These data indicate that HNA-3 mismatches, and in particular HNA-3a, may not increase the risk of alloimmunization in Thais.

In this study, we found no case of AMR in kidney-transplant patients associated with anti-HNA-3a or HNA-3b. This contrasts with Key *et al*,[17] who reported that HNA-3a antibodies were implicated in cases of kidney-transplant rejection. In that study, all kidney rejection patients (n = 7) were parous females with a history of the previous transfusion and transplantation and they also had performed anti-HNA-3a antibodies. Thus, a previous pregnancy constituted a major risk factor for the development of HNA antibodies and the presence of preformed anti-HNA-3a may affect transplant outcome. Even in pregnancy, the frequencies of HNA-3a antibodies are very low (0.26%), and less than 1.0% in individuals on the transplant waiting list.[17] However, HLA-class I and/or class II antibodies were

found in kidney transplant patients. Taken together, these findings indicate that HNA-3a antibodies are not a major factor in graft rejection.

Recently, Lopes and colleagues[21] demonstrated the possible role of anti-HNA-3b in cases of NAIN suggesting the important role not only of anti-HNA-3a but also anti-HNA-3b antibodies. Although high incompatibility in HNA-3b (79%) was found in our cohort, HNA-3b antibodies were also not detectable. However, HNA-3b antibodies could trigger neutrophil aggregation and can be easily identified by GAT assay.[12,21] Previous studies demonstrated that around 20% of pregnant women have the possibility of incompatibility with HNA-3b, and yet the immunization rate for anti-HNA-3b (0.5%) was much smaller than for anti-HNA-3a (7%).[12] Conversely, the rate of alloimmunization for HNA-3b seems to be higher in Brazil (1.07%).[21] Accordingly, the HNA-3b antigen may be less immunogenic than the HNA-3a antigen.

HNA-3 is located on CTL-2, a highly glycosylated protein with a broad cellular and tissue distribution. Two different isoforms CTL2-P1 and CTL2-P2 are identified which differ among different tissues. Whereas the kidney express both isoforms, the spleen, and heart only express CTL2-P1 or CTL-P2, respectively.[3] In addition, Bayat and colleagues[6] demonstrated that neutrophils only express CTL2-P1, whereas endothelial cells express CTL2-P1 and CTL2-P2 isoforms. Accordingly, only antibodies against CTL2-P1 could be identified, when antibody screening is performed agglutination assay using neutrophils as a target. In other words, antibodies against CTL-P2 may play a role in the pathomechanism of kidney rejection may overlook by this approach. Thus, further analysis such as antibody testing with endothelial cells or transfected cell lines carrying both isoforms may deepen our knowledge about the relevance of HNA-3 antibodies in graft rejection.

The screening and identification of HNA-3 alloantibodies have been hampered because of the lack of an appropriate monoclonal antibody against CTL-2 for use in a monoclonal-antibody-specific immobilization of granulocyte antigen assay. GAT remains the best technique for the detection of anti-HNA-3a and anti-HNA-3b antibodies.[12,20,22] However, this method needs fresh HNA-phenotype neutrophils, because it depends on the ability of these neutrophils to agglutinate. Recombinant HNA-3a and HNA-3b peptides to detect anti-HNA-3 are available; however, there are still some limitations due to their conformational changes.[23] Because of this, we decided to use the GAT together with the LabScreen Multi HNA kit to detect HNA-3 antibodies: both techniques yielded comparable results.

A limitation of our study was the small number of patients who were homozygous for HNA-3b, a genotype that might be expected to induce potent HNA-3a antibodies. Additional studies with larger sample sizes to assess the pathological significance and prevalence of HNA-3 antibodies in kidney transplantation and the mechanism of anti-HNA-3 implicated in kidney rejection in an animal model are suggested to confirm the risk-association of antibodies. Moreover, in the future, we should consider the presence of HNA-3 antibodies when lymphocyte cross-matches are positive but HLA antibodies are negative: this may be due to the expression of HNA-3 antigen on lymphocytes.

Conclusion

Our data provide evidence that HNA-3 incompatibility in kidney-transplant recipients with AMR is high (36.7%). Despite this, we could not find HNA-3 antibodies. These findings indicate that HNA-3 antibodies are less involved in kidney transplant rejection.

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Author contribution

P. M. K. performed the experiments, acquisition of data, analysis and interpretation of data and wrote the article. A. R. and C. L. contributed to reagents/ materials and were responsible for the critical revision of the article. C. C. O. was responsible for critical revision. P. S. contributed to design, reagents/ materials, analysis and interpretation of data and wrote and edited the article.

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