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Detection of anti-MICA*010 in plasma of kidney transplant patients with pathologically confirmed antibody-mediated rejection

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KEYWORDS
Recombinant protein;
Protein purification;
MICA*010;
Antibody-mediated rejection;
Kidney transplantation.

ABSTRACT

The screening for major histocompatibility complex class I- chain related A (MICA) antibodies in pre- and post-kidney transplantation is crucial to minimize the risk of immunological complications and antibody-mediated kidney rejection (AMR). However, the conventional method for detecting anti-MICA antibodies does not include all relevant MICA, especially MICA*010, a predominant MICA allele in northeastern Thais (NETs). Moreover, a significant number of patients have been diagnosed with AMR despite the absence of detectable antibodies. We hypothesized that anti-MICA*010 might be responsible for rejection in such instances. The household recombinant MICA*010 protein was successfully produced by HEK293 T cells and purified by using a cobalt-based magnetic bead with the percent recovery of 88.994. The ELISA testing using purified MICA*010 proteins was performed on plasma samples from 122 patients who were pathologically confirmed with AMR. Our findings showed that 21 samples (17.21%) in AMR patients exhibited the presence of anti-MICA*010. The flow cytometry showed a concordant result with ELISA. Among these samples with anti-MICA*010, the co-existing anti-HLA antibody was observed in one sample. Furthermore, there were six patients (4.9%) with positive for ELISA were negative for both anti-HLA and other anti-MICA antibodies as identified by Lab Screening Mix (LSM) Luminex test suggesting the importance of anti-MCIA*010 detection. Although further research with the comprehensive evaluation regarding the involvement of anti-MICA*010 in the kidney rejection episode is still needed, the present study is the first to highlight the presence of this antibody, which could offer valuable information for antibody detection in the future.

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Introduction

Kidney transplantation provides superior benefits in both survival and quality of life compared to undergoing regular dialysis treatment. Despite the advantages of kidney transplantation, the primary hindrance to its success is the incompatibility between the donor's tissue and the recipient's immune responses, which could result in graft failure via cellular-mediated rejection (CMR) or antibody-mediated rejection (AMR). AMR is caused by the preformed and/or de novo antibodies of patients that are specifically bound to the incompatible antigenic determinant found on the kidney tissue. In addition to the human leukocyte antigen (HLA), the major histocompatibility complex class I- chain related A (MICA) is considered one of the most important non-HLA antigenic molecules that play a critical role in kidney transplant rejection[1,2]. MICA is generally non-expressed in normal tissue. However, the upregulation of the MICA protein is found in various conditions including viral and bacterial infections, heat shock and DNA damage responses, oncogenic transformations, autoimmune conditions as well as inflammation[3]. Moreover, MICA has been found to be expressed on the renal tubular epithelial cells in response to ischemia-reperfusion injury, which might be occurred during the transplantation process, through the HIF-1α pathway[4].

Due to the extensive polymorphism of MICA proteins, antibody screening is necessary and routinely performed in pre- and post-kidney transplantation to investigate the cause of kidney rejection. As the MICA genotype is varied between ethnic groups, the antibodies produced by the recipient may be different. Several technologies have been developed for anti-MICA detection, such as ELISA and Luminex®[5,6]. Although the Luminex is widely acknowledged as the preferred method for MICA antibody testing, its capability to detect antibody specificity is limited which depends on the recombinant MICA protein immobilized on the beads. As a result, certain MICA alleles, such as MICA*010, are not included by this approach. The MICA*010 has been noted as an unexpressed MICA allele for decades due to a single amino acid substitution (proline for arginine) at position 6 in the first ß-strand of the α1 domain[7]. On the contrary, the study in our group found that the MICA*010 is certainly expressed on the cell surface (in preparation). In addition, this MICA allele is predominantly found (18.2%) in the northeastern Thais (NETs) and was shown to be significantly different from those of the Japanese and Caucasian populations (10.8% and 5.0%, respectively)[8]. Moreover, there are a large proportion of patients who have pathologically confirmed AMR with no detectable antibodies against HLA and/or MICA[9-11]. This indicates the inadequacy of current methods to comprehensively identify the relevant antibody that might contribute to kidney rejection. This information sparks the idea regarding the relevance of anti-MICA*010 in the AMR mechanism, particularly in cases of AMR without detectable antibodies, and requires further elucidation.

In the present study, the recombinant MICA*010 protein was produced and purified for the purpose of anti-MICA*010 detection in the patient's plasma. The plasma samples collected from histopathological proven AMR patients were screened for the presence of anti-MICA*010 by ELISA. Although the association between anti-MICA*010 and kidney transplant outcome is still unclear, our finding is the first study to demonstrate the existence of anti-MICA*010 in the plasma of patients which provides valuable information for further studies.

Materials and methods

Plasma samples

The 122 left-over plasma samples of patients who were pathologically diagnosed with antibody-mediated rejection (AMR) from Srinagarind Hospital were tested for the presence of the anti-MICA*010 antibody (the project was approved by Khon Kaen University Ethics

Committee for Human Research, HE601437). AMR was determined based on the classification guidelines provided by Banff 2017. To diagnose AMR, it was necessary to meet at least two out of the following three criteria: (i) histologic evidence of acute tissue injury; (ii) evidence of current/recent antibody interaction with vascular endothelium; and (iii) serologic evidence of DSA to HLA or other antigens, C4d staining or expression of validated transcripts/classifiers.

Construction of MICA*010 containing plasmid

The plasmid DNA carrying a soluble form of MICA*010 protein (sMICA*010) was generated by introducing a stop codon into the MICA*010 in the front of the transmembrane domain. MICA*010 was amplified from human genomic DNA (3 healthy subjects who possess homozygous MICA*010 that were obtained from the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University with informed consent (HE571283) by the PCR method. The sequences of forward and reverse primers for MICA*010 amplification are shown in table 1. The amplification cycles were 94°C for 2 min, then 30 cycles of 95°C for 30 sec, 60°C for 45 sec and 65°C for 2 min followed by 65°C for 10 min using the thermal cycler with 2 mM of $MgSO_4$. The PCR products were run in an agarose gel electrophoresis and subsequently purified from gel using the GFX gel band purification kit in accordance with the manufacturer's guidelines. The purified sMICA*010 gene was then ligated to the expression vector (pcDNA™3.1/V5-His TOPO™ TA Expression Kit) according to the manufacturer's instructions. The MICA*010 carrying plasmid was then transformed into a bacterial cell line (TG1) to amplify the plasmid DNA and purification. The plasmid DNA that carries the sMICA*010 gene was purified and verified by PCR-SSP[12,13] and nucleotide sequencing.

Table 1 Nucleotide sequences and annealing temperature of forward and reverse primer for soluble MICA*010 construction

Primer name	Primer sequence (5'→ 3')	Annealing temperature
F_Stop_sMICA010	CACC*ATGGGGCTGGGCCCGGTCTTCCTGCTTCTGGCTGGCATCT TCCCTTTTGCACCTCCGGGAGCTGCTGCTG*<u>**AGCCCCACAGTCT TCCTT**</u> (92 bases, binding site = 18 bases)	60 °C
R_Stop_sMICA010	CTA<u>ATGGTGGTGGTGATGATG</u>**CTGAAGCACCAGCACTTT** (39 bases, binding site = 18 bases)	60 °C

Note: Bold letters = binding site, Italics = leader sequence, Underline character = 6X-Histidine, CACC = TOPO sequence (necessary to integrate the interested gene into TOPO vector)

Transfection of sMICA*010 plasmid to HEK 293T cells

The sMICA*010 carrying plasmid was transfected into HEK 293T cells, which were cultured overnight in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% pen-strep antibiotics. X-treme GENE HP DNA transfection reagent was used to transfect the plasmid following the manufacturer's instructions. As a control, an empty plasmid (pcDNA 3.1) was also transfected (MOCK). A household plasmid encoding the green fluorescence protein (GFP) was transfected in parallel to assess the transfection efficiency. The

transfection efficiency was investigated after 48 h of transfection using flow cytometry. The transfected cells were collected 72 h post-transfection for purification of soluble MICA*010 protein.

Recombinant MICA*010 protein collection and purification

The transfected HEK 293T cells were detached from the cell culture plates by repetitive up-and-down pipetting until all cells were completely detached from the culture plate. The cells were lysed by using the lysis buffer and subsequently centrifuge at 8,000 rpm for 10 min to provide an appropriate supernatant for the purification. The Dynabeads™ His-Tag Isolation & Pulldown utilizes cobalt-based immobilized metal affinity chromatography (IMAC) chemistry on the magnetic beads to specifically bind histidine-tagged proteins. The purification steps including MICA*010 protein binding to the cobalt-immobilized magnetic bead followed by three times of washing, and two times of elution step. In each step, the supernatant was carefully collected by aspiration at 2 min after the tube was placed on the magnet. All fractions, including crude, flowthrough, wash 1 and 2, eluate 1 and 2, were collected and used to verify the efficiency of protein purification. The purification process is shown in figure 1. The protein purity was investigated by running the SDS-PAGE and western blot.

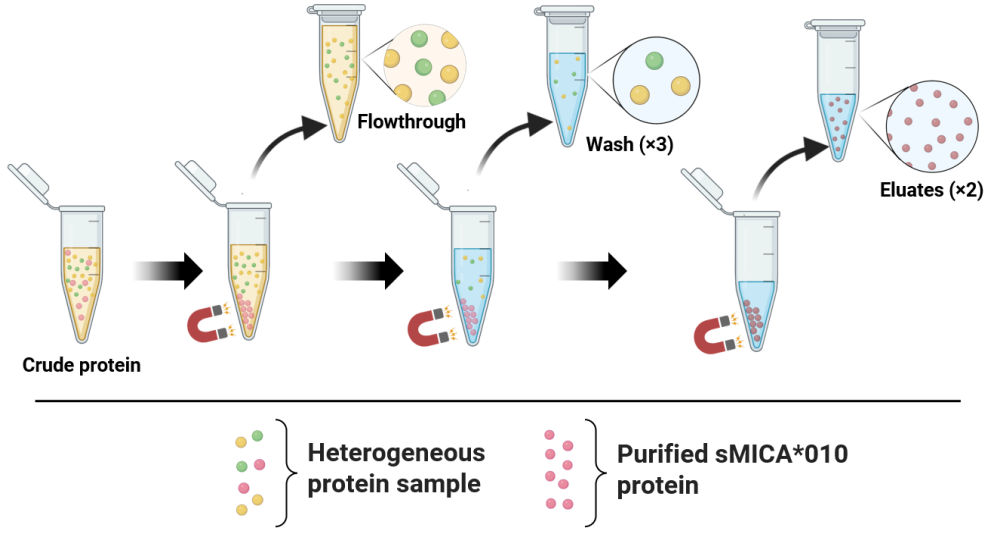


Figure 1 The illustration of protein purification processes using Dynabeads™ His-Tag Isolation & Pulldown. The purification steps including MICA*010 protein binding to the cobalt-immobilized magnetic bead, washing step and elution step.

Determination of the purity of purified MICA*010 protein by western blot

The purity of the purified protein was assessed by using western blot method. The concentration of all fractions was determined before running the western blot by using the BCA assay kit according to the manufacturer's instructions. The percent recovery of a protein production process was calculated by comparing the amount of the target protein recovered at the end of the process to the amount of protein that was initially present in the starting crude. The formula for calculating the percent recovery is as follows:

% Recovery = (Amount of pure MICA*010 protein recovered/Amount of starting crude protein) x 100%

All fractions, including crude MICA*010 protein, flowthrough, wash fraction and eluates, obtained from the purification process were run in the 12.5% polyacrylamide gel with 150 volts for 90 min. The gel was cut and blot to the polyvinylidene difluoride (PVDF) membrane to transfer all the protein to the membrane using semi-dry method (300 volts, 80 min). After that the membrane was washed 1 time with 0.1% Tween-20 in Tris-Buffered Saline (TBS-T). The membrane was blocked with 5% skim milk prepared in 0.1% TBS-T at RT for 1 h. The membranes were separately incubated with 0.5 µg/mL of anti-MICA (clone WJ-1, household) or anti-6X Histidine (Biolegend, SD, CA) for overnight at 4 °C with shaker. The membranes were washed 3 times with 0.1% TBS-T and probed with the secondary antibody (HRP-labelled goat anti-mouse IgG (Biolegend, SD, CA) in the dilution of 1:1,000 prepared in blocking buffer) at RT for 1 h. After this, the membranes were washed 3 times and detected by using ECL western blot detection kit (Cytiva, MA, USA).

Detection of anti-MICA*010 in patients' plasma using ELISA

The ELISA method was developed for anti-MICA*010 detection. In brief, the ELISA plate was coated with 3 µg/well of purified sMICA*010 protein prepared in carbonate-bicarbonate buffer, pH 9.8 for overnight at 4°C. After washing with 0.2% PBS-T to remove unbound proteins, 300 µL of blocking buffer (5% skim milk prepared in 0.1% PBS-T) was added to each well and subsequently incubated at 37°C for 2 h in order to block free area of the ELISA plate. 100 µL of diluted plasma samples (dilution of 1:100 with 3% skim milk) were added and incubated at 37ºC for 1 h. Subsequently, washed and added the HRP-tagged goat anti-human IgG (Biolegend, SD, USA) at the dilution 1:5,000 in 2% skim milk for 1 h at 37ºC. Then, the conjugated antibody was removed by flicking the plate over a sink and after 3 washing with washing buffer, 100 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added for 1 h in the dark at room temperature. The absorbance was measured at 450 nm by microplate reader (Sunrise™, Tecan, Männedorf, Switzerland). The cut-off value was defined as mean + 2SD of the absorbance (OD450) obtained from the plasma of 50 healthy donors. The positive or negative was defined by using the established cut-off value.

Establishment of flow cytometry to verify the presence of anti-MICA*010 in patients' plasma

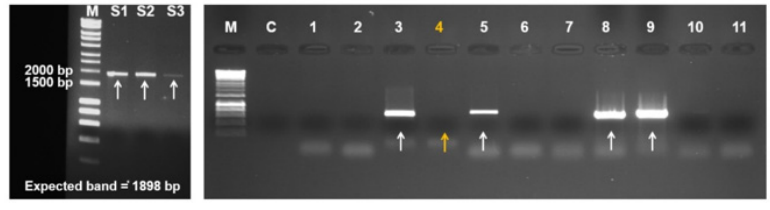
The experiment was conducted using HEK 293T cells transfected with the MICA*010 plasmid (intact form) and simultaneously transfected with an empty plasmid (pcDNA 3.1) as a mock control. The cells were harvested 48 h after transfection, washed with sterile 1X PBS, and blocked with 500 µL of blocking buffer (consisting of 20% AB normal serum and 2% FBS in sterile 1X PBS) for 15 minutes. After washing, each dilution of the patient's plasma ranging from 0 (PBS buffer) to 1:100 was separately incubated with the MICA*010 or MOCK transfectants on ice for 1 hour. For the pioneering study, three plasma samples from healthy donors and two from kidney transplant patients that showed positive result from ELISA (no. DSA20 and DSA23) were used. The cells were then washed, and 5 µL of goat anti-human IgG labeled with Allophycocyanin (APC) was added for a 30-minute incubation on ice. After final washing with 1X PBS, the cells were analyzed using flow cytometry. The mean fluorescence intensity (MFI), which is a measure of the amount of fluorescence emitted by a sample, was measured for each transfectant after incubation with the plasma samples. The MFI values obtained for the MICA*010 and MOCK transfectants were compared to determine the presence of anti-MICA*010 in the patient's plasma.

Results

Cloning of the MICA*010 gene to the expression vector for protein production

To obtain the soluble form of the MICA*010 protein, MICA*010 was amplified from human genomic DNA (3 subjects from our previous study who were identified as a MICA*010/010 genotype

by a PCR-SSP). As shown in figure 2A, the expected PCR product size appeared at 1898 bp which was found in all three samples. PCR products were cut and purified. Then, cloning into the expression vector (pcDNA 3.1) and subsequently verified by PCR-SSP (Figure 2B). The interpretation of the PCR-SSP for MICA genotyping dictates that the MICA*010 allele should have positive bands in reactions 3, 5, 8, and 9[12,13]. Additionally, homozygous MICA*010 individuals must exhibit negative bands when tested with primer mixture 4, which is specific for the MICA*019 allele. This result indicates the appearance of the MICA*010 gene inserted in the purified plasmid DNA extracted from the bacterial clone. The result was finally confirmed by DNA sequencing. The amino acid sequence of three extracellular domains (exon 2, 3 and 4) of MICA*010 inserted in the plasmid DNA was identical to that of the sequence retrieved from the HLA/MICA database (http://hla.alleles.org/data/mica.html; Release version 3.44.0, 13 January 2022) as shown in figure 2C. All of these findings support the successful cloning of the MICA*010 (exons 2-4) into the expression vector pcDNA 3.1 for the production and purification of protein.



(A)

(B)

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                         5         15         25         35         45
MICA*010 (from database) EPHSLPYNLT VLSWDGSVQS GFLAEVHLDG QPFLRYDRQK CRAKPQGQWA
sMICA*010 (Sequencing)   EPHSLPYNLT VLSWDGSVQS GFLAEVHLDG QPFLRYDRQK CRAKPQGQWA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                        55         65         75         85         95
MICA*010 (from database) EDVLGNKTWD RETRDLTGNG KDLRMTLAHI KDQKEGLHSL QEIRVCEIHE
sMICA*010 (Sequencing)   EDVLGNKTWD RETRDLTGNG KDLRMTLAHI KDQKEGLHSL QEIRVCEIHE

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                       105        115        125        135        145
MICA*010 (from database) DNSTRSSQHF YYDGELFLSQ NLETEEWTVP QSSRAQTLAM NVRNFLKEDA
sMICA*010 (Sequencing)   DNSTRSSQHF YYDGELFLSQ NLETEEWTVP QSSRAQTLAM NVRNFLKEDA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                       155        165        175        185        195
MICA*010 (from database) MKTKTHYHAM HADCLQELRR YLESSVVLRR TVPPMVNVTR SEASEGNITV
sMICA*010 (Sequencing)   MKTKTHYHAM HADCLQELRR YLESSVVLRR TVPPMVNVTR SEASEGNITV

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                       205        215        225        235        245
MICA*010 (from database) TCRASSFYPR NIILTWRQDG VSLSHDTQQW GDVLPDGNGT YQTWVATRIC
sMICA*010 (Sequencing)   TCRASSFYPR NIILTWRQDG VSLSHDTQQW GDVLPDGNGT YQTWVATRIC

                    ....|....| ....|....| ....|....| ....|...
                       255        265        275        285
MICA*010 (from database) RGEEQRFTCY MEHSGNHSTH PVPSX----- -------X
sMICA*010 (Sequencing)   RGEEQRFTCY MEHSGNHSTH PVPSESAGAS ASSPPPLX
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(C)

Figure 2 The analysis of sMICA*010 clone (A) The PCR bands investigated by an agarose gel electrophoresis. The amplified MICA gene from each individual; M, DNA marker; S1, subject 1; S2, subject 2; S3, subject 3. (B) The PCR-SSP result of the MICA*010 inserted plasmid using 11 primer mixtures, M, DNA marker; C, negative control; number 1 – 11 represent the number of primer mixtures used for the PCR-SSP. (C) The alignment of the amino acid sequence of the MICA*010 protein. The amino acid sequence of the MICA*010 retrieved from the HLA/MICA database aligned with the plasmid DNA sequencing. Exons 2, 3 and 4 were highlighted in the yellow, red and green boxes, respectively.

Production and purification of recombinant MICA*010 protein and verification of protein purity

The plasmid DNA encoding green fluorescence protein (GFP) was simultaneously transfected to examine the transfection efficiency. The GFP transfectant revealed the high efficiency of plasmid transfection to the HEK 293T cells with 70-90% of the transfection efficiency as measured by flow cytometry (Figure 3A). After 72 h of transfection, the sMICA*010 transfected cells were collected. All fractions including crude, flowthrough, wash and eluates were collected and stored at -20 °C for further use. The protein concentration was determined by the BCA protein assay (Thermo Scientific, MA, USA). Evidently, the purification process provides a high percent recovery at 88.994%. In addition, the protein purity was assessed using SDS-PAGE and western blot. After probing with the anti-6X histidine or anti-MICA (clone WJ-1), the MICA-specific band appeared at around 50 kDa in both antibody clones. The Coomassie blue staining revealed more purity of the protein band when compared to the crude mock and crude MICA*010 protein (Figure 3B). It should be noted that, based on the principle of the protein purification kit that the His-tagged protein binds to the cobalt ions on the magnetic beads, other proteins with exposed histidine residues that are not part of a His-tag may also bind non-specifically to the beads. The non-specific protein was found after staining with both antibodies (Figures 3C and 3D). However, most of the protein in the eluates is occupied by the recombinant MICA*010 protein as demonstrated by the density of the band after staining with the specific anti-MICA antibody. Alternatively, these multiple bands stained by both antibodies could represent different forms of glycosylations. This indicated the applicability of the purified recombinant protein for further step.

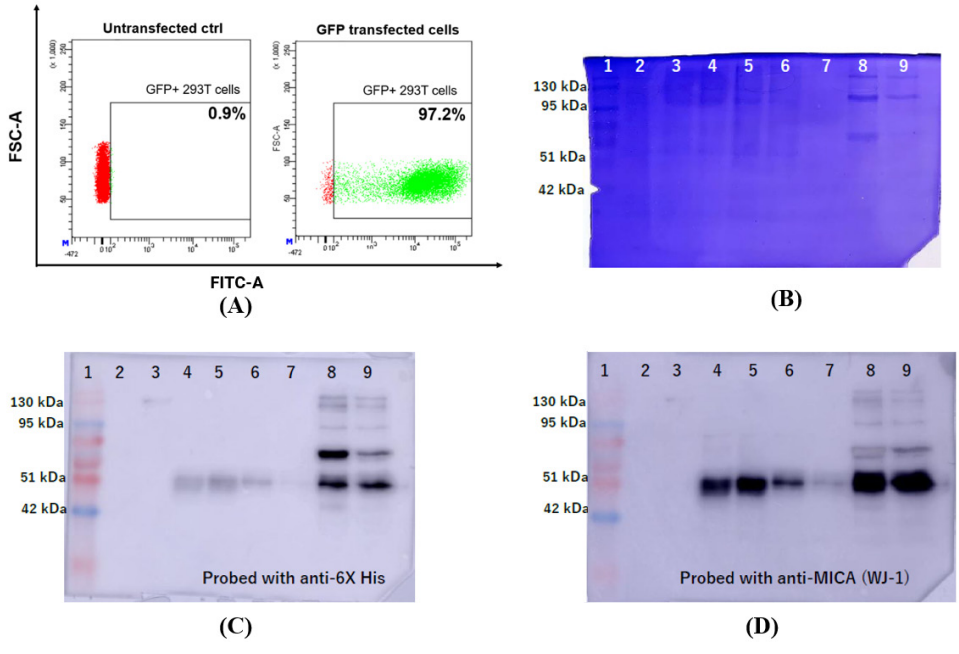

Figure 3 Evaluation of MICA*010 transfection and protein purity after protein purification process. (A) The transfection efficiency assessed by GFP transfection. (B) The polyacrylamide gel stained with Coomassie blue R-250 solution. (C) The western blot analysis which probed with anti-6X His. (D) The western blot analysis which probed with anti-MICA, clone WJ-1. All fractions of proteins were investigated under denaturing conditions in the presence of dithiothreitol (DTT) Lane 1, protein marker; lane 2, crude Mock; lane 3, crude ß-galactosidase; lane 4, crude MICA*010; lane 5, flowthrough; lane 6, wash 1; lane 7, wash 2; lane 8, eluate 1 and lane 9, eluate 2.

Detection of anti-MICA*010 in patients' plasma using developed ELISA method

To detect anti-MICA*010 in patients' plasma, the ELISA method was developed and tested on 50 healthy donor plasma samples to determine the cut-off value. The mean and standard deviation of the absorbance (OD450) from healthy donor plasma samples were calculated as 0.264 and 0.124, respectively. The cut-off value, which is defined by mean + 2SD of the healthy subjects, was determined to be 0.511. Plasma samples from 122 kidney transplant patients with AMR were then tested using this method. Among all samples, 21 samples (17.21%) showed an OD greater than the cut-off value (Figure 4A), indicating the presence of anti-MICA*010. The available data about the *MICA* genotype of both the donor and patient, as well as the anti-HLA profile of these 21 samples, were considered. The data regarding the anti-HLA and -MICA profiles of these samples was kindly obtained from the blood bank, Srinagarind Hospital. The complete LSM data were available for nine samples, which included both anti-HLA and anti-MICA antibodies. Additionally, there were ten samples that only had anti-HLA data, while LSM data were unavailable for two samples for either anti-HLA or anti-MICA antibodies. The data of Lab Screening Mix (LSM) Luminex test revealed two plasma samples, among 21 positive ELISA, showed positive signals. One sample had a positive result for bead no. 68, which detected MICA*001, *004, *012, *018 and 027, while the other sample had a positive result for bead no. 97, which detected MICA*002, *007, *009, *017 and *019. Although the incomplete dataset of the LSM Luminex test possess a limitation to our study, the identification of co-existing anti-MICA and anti-HLA class I and II antibodies in a particular sample (DSA23) suggests their involvement in the episode of kidney rejection through an antibody-mediated mechanism (Figure 4B).

Verification of anti-MICA*010 in patients' plasma using flow cytometry

To verify the ELISA result, flow cytometry was additionally performed. The limitation of this study is that the HEK 293T cells used in the experiment have internal MICA expression, which makes it difficult to accurately identify the true signal of anti-MICA*010 positive. To address this issue, the gap MFI signal (ΔMFI) between the MICA*010 transfectant and MOCK control (ΔMFI = $MFI_{MICA*010} - MFI_{MOCK}$) was a more accurate measure. As a result, by considering the ΔMFI (Figure 4C), it was clear that the ΔMFI found in the patient samples was higher than that of the healthy donors. The flow cytometry results showed the concordant pattern with the ELISA, verifying the appearance of an anti-MICA*010 in the plasma sample. However, this is a pioneering study, and further research with a greater number of samples is required. Moreover, to avoid the interference of the internal MICA expression, the mammalian cell line without MICA expression is recommended.

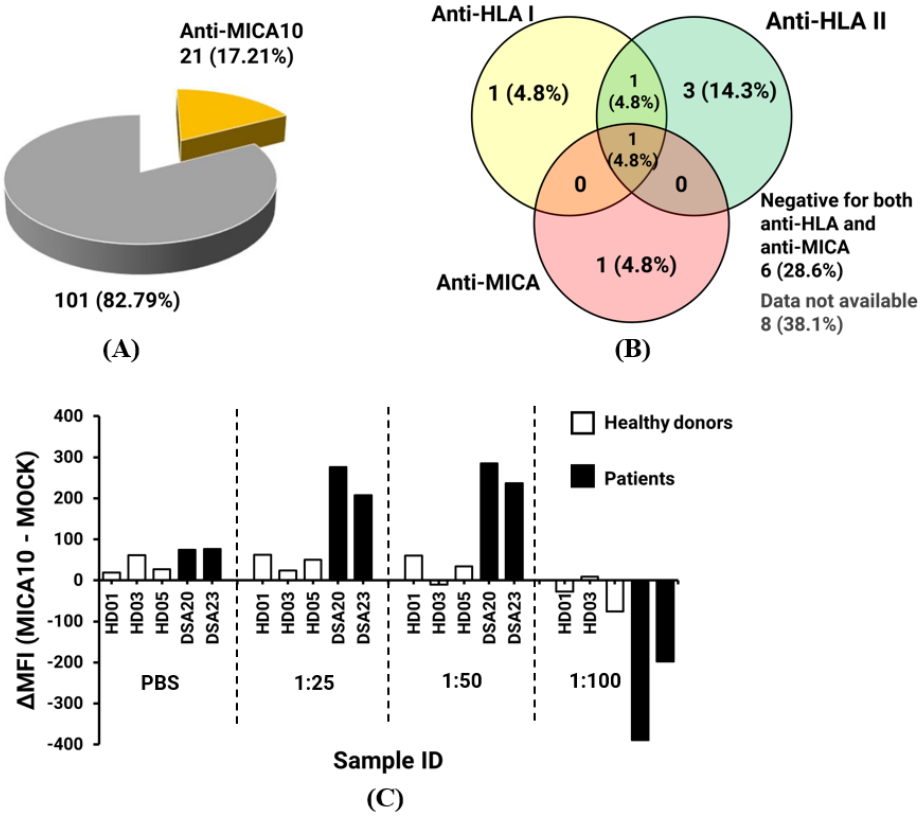

Figure 4 The detection of anti-MICA*010 in patient's plasma. (A) The percentage of the anti-MICA*010 detected by the developed ELISA method. (B) The distribution of anti-MICA and -HLA antibodies among the 21 plasma samples that were positive for ELISA. According to the available LSM data set, among 21 samples analyzed, one sample exhibited solely anti-HLA class I, three samples displayed anti-HLA class II, one sample demonstrated the presence of both anti-HLA class I and class II, one sample exhibited solely anti-MICA, one sample revealed the co-existence of anti-MICA along with anti-HLA class I and class II antibodies, six samples did not exhibit either anti-MICA or anti-HLA antibodies and data was not available in eight samples. (C) The ΔMFI of the anti-MICA*010 detection using flow cytometry presented in different plasma dilution including PBS (diluent), 1:25, 1:50 and 1:100. HD; healthy donor, DSA; patient plasma.

Discussion

Recently, the importance of the MICA protein in kidney transplantation has been acknowledged and extensively studied for its role in organ rejection. Studies suggest that anti-MICA antibodies binding to MICA molecules on endothelial cells in transplanted kidneys may affect transplantation outcomes[14,15]. The identification of anti-MICA antibodies prior to and after kidney transplantation relies on the Luminex technology, which can detect numerous MICA-specific antibodies, except the antibody against MICA*010. The antibody specific to this MICA allele is hypothesized to be involved in kidney rejection as it showed a high frequency in NETs population. To obtain purified protein for downstream applications, a six-histidine tag was added to the C-terminal of the soluble MICA*010 protein. The purification was performed by using Dynabeads™His-Tag Isolation and Pulldown, which

is the optimized cobalt-based immobilized metal affinity chromatography (IMAC). The magnetic beads were coated with the cobalt ion that has a higher binding affinity for the His-tag than nickel. This higher affinity can be attributed to the stronger interaction between cobalt and the imidazole ring of histidine residues, which provide higher purity and yield compared to nickel-based resins[16,17]. The purification kit enables the pursuit of MICA*010 protein in HEK 293T cells by reducing the background contamination of other irrelevant cellular proteins. Despite utilizing the His-tag purification method, there were still other protein bands visible on the stained polyacrylamide gel. This may be attributed to other cellular proteins with exposed histidine residues that are not part of the his-tag, which tend to bind non-specifically to the magnetic beads. Alternatively, these multiple bands stained by both antibodies against MICA*010 and histidine could represent different forms of glycosylations. Nonetheless, western blot analysis indicated that the MICA*010 protein occupied the majority of the protein in the eluates when the specific antibody against the MICA protein was employed. Additionally, the purification method employed in this study successfully retrieved the desired his-tagged MICA*010 protein, as evidenced by the desirable percent recovery, exceeding 80% in this research. Furthermore, to eliminate the influence of additional impure proteins that might lead to non-specific binding and erroneous positive results in the anti-MICA*010 detection, the crude protein acquired from the MOCK control was utilized in each experiment.

Our study found that the MICA*010 allele is certainly expressed on the cell surface (in preparation), suggesting its potential contribution to kidney transplant outcomes. This study aimed to investigate this contribution and found that patients with AMR exhibited the presence of anti-MICA*010 through the ELISA method. Results from flow cytometry showed a similar trend to the ELISA findings, where the ΔMFI in patient plasma was higher than that of healthy donors. Out of the 21 samples that were tested positive for anti-MICA*010 via ELISA, one sample displayed the co-existence with anti-HLA class I and class II. Previous studies have shown that the presence of both anti-HLA and anti-MICA antibodies in kidney transplant patients is associated with a longer time to reach optimal serum creatinine level after transplantation[18]. Meanwhile, six patients with the positive OD from ELISA were tested negative for both anti-HLA and other anti-MICA antibodies. This information suggests that monitoring for anti-MICA*010 should be beneficial, particularly in the instances where the patient has been diagnosed with AMR. This data indicate that the simultaneous detection of antibodies targeting both HLA and non-HLA antigens (especially of MICA) may provide a more thorough evaluation of the patient's immune responses to the kidney allograft and may assist in the categorization of immunological risk. The limitation of this study with respect to the identification of anti-MICA*010 through flow cytometry was the internal HLA-I and MICA expressions found in the HEK 293T cells. However, this could be addressed by employing the MOCK control, which would be sufficient to exclude such expression. To obtain more reliable results regarding the presence of anti-MICA*010, it is recommended to perform transfection in HLA and MICA deficient mammalian cell lines. There is a study that proposed the method to eliminate the expression of *HLA* and *MICA* gene in the HEK 293T cells through CRISPR/Cas9 system[19]. Moreover, the HLA class I and MICA/B null HEK 293T panel expressing single MICA alleles has been reported to provide beneficial in anti-MICA antibodies detection[20]. This pioneering study suggested the presence of anti-MICA*010 in the plasma of AMR patients which could be served as an initial point to emphasize the importance of this antibody in the future. However, for more understanding of the clinical relevance of anti-MICA*010 in kidney rejection, increasing patient numbers and a comprehensive investigation that includes other relevant factors are also necessary to consider thoroughly.

Conclusion

The anti-MICA plays an essential role in kidney rejection. Therefore, comprehensive antibody detection is crucial for minimizing the immunological risk and help assessing the cause of AMR. The MICA*010 allele has been found predominantly in NETs and was hypothesized to be involved in the AMR episode. In this study, we successfully produced the recombinant MICA*010 protein and used to establish the ELISA method for anti-MICA*010 detection. Our findings also illustrate the presence of anti-MICA*010 in the plasma of AMR patients. Moreover, the co-existence of anti-MICA*010 and anti-HLA antibodies was observed. The involvement of this antibody in the occurrence of AMR is still unclear. However, this research could be a groundbreaking to emphasize the significance of this antibody in future studies.

Take home messages

The existence of anti-MICA*010 in plasma samples of patients with AMR was demonstrated, which paves the foundation regarding the possible role of this antibody on the AMR episode and necessitates further comprehensive investigation.

Conflicts of interest

The authors declare no conflict of interest.

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