MICB Allele Genotyping on Microarrays by Improving the Specificity of Extension Primers

In-Cheol Baek¹, Jung-Pil Jang¹, Eun-Jeong Choi², Tai-Gyu Kim¹,²*

¹ Department of Microbiology, College of Medicine, The Catholic University of Korea, Seoul, Korea, ² Hematopoietic Stem Cell Bank, College of Medicine, The Catholic University of Korea, Seoul, Korea

* kimtg@catholic.ac.kr

Abstract

Major histocompatibility complex (MHC) class I chain-related gene B (MICB) encodes a ligand for activating NKG2D that expressed in natural killer cells, γδ T cells, and αβ CD8⁺ T cells, which is associated with autoimmune diseases, cancer, and infectious diseases. Here, we have established a system for genotyping MICB alleles using allele-specific primer extension (ASPE) on microarrays. Thirty-six high quality, allele-specific extension primers were evaluated using strict and reliable cut-off values using mean fluorescence intensity (MFI), whereby an MFI >30,000 represented a positive signal and an MFI <10,000 represented a negative signal. Eight allele-specific extension primers were found to be false positives, five of which were improved by adjusting their length, and three of which were optimized by refractory modification. The MICB alleles (*002:01, *003, *005:02/*010, *005:03, *008, *009N, *018, and *024) present in the quality control panel could be exactly defined by 22 allele-specific extension primers. MICB genotypes that were identified by ASPE on microarrays were in full concordance with those identified by PCR-sequence-based typing. In conclusion, we have developed a method for genotyping MICB alleles using ASPE on microarrays; which can be applicable for large-scale single nucleotide polymorphism typing studies of population and disease associations.

Introduction

The product of major histocompatibility complex (MHC) class I chain-related gene B (Gene ID: 4277; MICB) is expressed as three extracellular domains, a transmembrane segment, and a carboxy-terminal cytoplasmic tail [1]. The flexible extracellular domains—α1, α2, and α3—together serve as a ligand for activating NKG2D that is expressed on natural killer (NK) cells, γδ T cells, and αβ CD8⁺ T cells [2]. As shown in the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla), over 40 polymorphic alleles have been found in exon 2 to 6 of MICB. Specific polymorphisms are associated with autoimmune diseases, cancer, and infectious diseases, as well as with the success of hematopoietic stem cell transplantation. For example, MICB*004 allele is significantly associated with susceptibility to rheumatoid arthritis in Spanish
Caucasians [3], MICA’005:02 allele is negatively associated with cervical cancer in the Thai population [4], and MICA’008 allele (designated as MICA’0106 allele in 2004) is positively associated with ulcerative colitis in the Han Chinese population of central China [5]. MICA’008 allele is also associated with a reduction in severity of dengue fever [6]. In addition, MICA’008 and ’002 alleles are associated with susceptibility to celiac disease in Europeans [7]. Moreover, recipients in need of a transplant had significantly improved survival probabilities when MICA and MICA alleles matched between the donor and recipient [8].

In previous studies, genotyping MICA alleles has been carried out using polymerase chain reaction with sequence-specific primers (PCR-SSP) and PCR-sequence-based typing (PCR-SBT) [9, 10]. Methods for detecting single nucleotide polymorphisms (SNPs) on a microarray platform include single base extension (SBE), allele-specific primer extension (ASPE), oligonucleotide ligation, allele-specific oligonucleotide direct hybridization, allele-specific cleavage of a flap endonuclease, and 5‘ nuclease assay [11–13]. In this study, we developed a high-throughput genotyping system for MICA alleles by optimizing allele-specific extension primers for ASPE on glass slides.

Materials and Methods

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood samples taken from 200 healthy Korean individuals (staff and students of the College of Medicine, The Catholic University of Korea) using an AccuPrep Genomic DNA Extraction Kit (Bioneer Corporation; Daejeon, Korea), following the manufacturer’s instructions. Briefly, the cells were lysed in 100 μL K buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl₂, 0.5% Tween 20, and 100 μg/mL Proteinase K) for 60 min at 56°C and then inactivated for 10 min at 95°C. After extraction, the DNA concentration was adjusted to 100 ng/μL and the resulting DNA samples were used as the PCR template in the genotyping assays.

In the UCLA International KIR and MICA Exchange Report program, 95 DNA samples for KIR Exchange (KDNA # 0021, 0038 ~ 0067, 0069 ~ 0084, 0087 ~ 0109, 0111 ~ 0118, 0120 ~ 0136) and 68 DNA samples for MICA Exchange (MICA#025 ~ 092) were used for MICA genotyping by PCR-SBT. Six samples among them were defined to be MICA’005:01, ’005:06 and ’024 alleles, which did not find in Korean population and included to establish MICA genotyping on microarrays (Table 1).

Ethics statement

The study was approved by the Catholic University Institutional Review Board (IRB), with written informed consent from all subjects (Institutional Review Board number: MC13SISI0126).

Preparation of microarrays

The matched control extension primers define as the N0 group were designed to match the MICA sequence without SNPs. Mismatched control extension primers were designed to contain artificial mismatches at the 1st, 2nd, 3rd, and 4th base from the 3’ ends of the N0 extension primers, and were called the N1, N2, N3, and N4 groups, respectively. Each group consisted of four primers containing an adenine, cytosine, guanine, or thymine (A, C, G, or T) as the mismatch at the 3’ end (Table 2).

Microarrays were prepared as previously described [15]. Each of the MICA allele-specific extension primers (50 μmol/L), which were designed based on the sequences of the MICA
polymorphisms with an additional 18 bp oligo(dT) spacer at the 5’ end, was dissolved in a buffer containing 350 mmol/L sodium bicarbonate (pH 9.0) (Tables 3 and 4). The primers were then spotted onto standard microscope glass slides coated with aldehyde (SCHOTT; Jena, Germany) using a MicroGridII (BioRobotics; Cambridge, MA, USA) controlled with an MCM-310 operating system. The primers were immobilized as NH2-modified oligonucleotides. Distilled water was used to hydrate the primers on the slides at 25°C for 1 h in a humid chamber, and then the slides were baked at 120°C for 1 h.

Next, to deoxidize the aldehyde residue, the slides were washed for 30 min, first with 0.2% sodium dodecyl sulfate (Sigma; Dorset, UK), followed by distilled water, and then with an NaBH4 solution containing 1 g NaBH4, 300 mL PBS, and 100 mL EtOH. Finally, the slides were rinsed with 0.2% sodium dodecyl sulfate followed by distilled water and then dried. The processed slides were stored at −70°C in a silica-gel box.

**Amplification of MICB**

Primer sets were designed to amplify MICB polymorphisms, which are present in exon 2, 3, 4, and 5 of the MICB gene. The forward primers were modified to have a phosphate group (P) at the 5’-end. The following primers were used in each single tube: sense 1, 5’-P-CAATGTGAA GTTAATTTCCAGGAAAG-3’ from positions 7369 to 7395; antisense 1, 5’-CCAGGGT CGGTACCTGTTCT-3’ (from positions 8230 to 8249) for MICB exon 2 and 3; sense 2, 5’-P-CTGTTCCCTGCATCTCCCTAGA-3’ (from positions 8765 to 8787); antisense 2, 5’-CCCATCTCCAGAAACTGTCCCC-3’ (from positions 9346 to 9368) for MICB exon 4 and 5 [14]. PCR was carried out in a reaction volume of 40 μL containing 500 ng genomic DNA, 1× buffer (60 mmol/L Tris-Cl, 15 mmol/L ammonium sulfate, 100 mmol/L MgCl2), 200 μmol/L dATP/dGTP/dCTP, 200 μmol/L dTTP/dUTP (dT:dU = 7:3), 4U Taq DNA polymerase.

| Sample no. | MICB alleles by PCR-SBT | Population |
|------------|-------------------------|------------|
| CHB-4      | *004:01:01              | Korean     |
| CHB-13     | *002:01:01              | Korean     |
| CHB-17     | *002:01:01              | Korean     |
| CHB-19     | *002:01:01              | Korean     |
| CHB-38     | *002:01:01              | Korean     |
| CHB-47     | *003                    | Korean     |
| CHB-70     | *005:02                 | Korean     |
| CHB-72     | *002:01:01              | Korean     |
| CHB-73     | *002:01:01              | Korean     |
| CHB-77     | *002:01:01              | Korean     |
| CHB-78     | *004:01:01              | Korean     |
| CHB-84     | *003                    | Korean     |
| KDNA # 0064 | *005:03              | Asian Indian |
| KDNA # 0124 | *005:02/*010           | Caucasian  |
| KDNA # 0128 | *005:02/*010           | Caucasian  |
| MICA#036   | *004:01                 | Black      |
| MICA#047   | *005:02/*010           | Hispanic   |
| MICA#88    | *005:02/*010           | Black      |

*CHB is abbreviation of the Catholic Hematopoietic Stem Cell Bank (http://www.chscb.com).
Table 2. Control extension primer set for optimal conditions of ASPE on microarrays.

| Groups according to mismatches from 3' ends | Names of control extension primers | Sequence (5'-3') | Region\(^{\dag}\) | Length (bp) | Tm (°C) \(^{†}\) | GC contents (%) |
|------------------------------------------|----------------------------------|----------------|----------------|--------------|----------------|----------------|
| N0 (matched)                             | A0 GAGAATGGGCAAGACCTCA           | 7623–7641       | 19             | 51.1         | 52.6           |
|                                          | C0 TGTGCAGTCAGGGTTTCTC           | 7472–7490       | 19             | 51.1         | 52.6           |
|                                          | G0 GCAGTGGGGGGATGTCCGT           | 8967–8985       | 19             | 57.6         | 68.4           |
|                                          | T0 TGGATGGTCAGCCCTTCT            | 7504–7522       | 19             | 53.2         | 57.9           |
| N1 (mismatched 1st base from 3'ends)     | A1 GAGAATGGGCAAGACCTCG           | 7623–7641       | 19             | 53.2         | 57.9           |
|                                          | C1 TGTGCAGTCAGGGTTTCAT          | 7472–7490       | 19             | 48.9         | 47.4           |
|                                          | G1 GCAGTGGGGGGATGTCCGA           | 8967–8985       | 19             | 55.4         | 63.2           |
|                                          | T1 TGGATGGTCAGCCCTTCG            | 7504–7522       | 19             | 55.4         | 63.2           |
| N2 (mismatched 2nd base from 3' ends)    | A2 GAGAATGGGCAAGACCTTA          | 7623–7641       | 19             | 48.9         | 47.4           |
|                                          | C2 TGTGCAGTCAGGGTTTCG           | 7472–7490       | 19             | 53.2         | 57.9           |
|                                          | G2 GCAGTGGGGGGATGTCCGG           | 8967–8985       | 19             | 59.7         | 73.7           |
|                                          | T2 TGGATGGTCAGCCCTTCAT           | 7504–7522       | 19             | 51.1         | 52.6           |
| N3 (mismatched 3rd base from 3' ends)    | A3 GAGAATGGGCAAGACCGCA          | 7623–7641       | 19             | 53.2         | 57.9           |
|                                          | C3 TGTGCAGTCAGGGTTTATC          | 7472–7490       | 19             | 48.9         | 47.4           |
|                                          | G3 GCAGTGGGGGGATGTCCATG          | 8967–8985       | 19             | 55.4         | 63.2           |
|                                          | T3 TGGATGGTCAGCCCTTACT           | 7504–7522       | 19             | 51.1         | 52.6           |
| N4 (mismatched 4th base from 3' ends)    | A4 GAGAATGGGCAAGACATCA          | 7623–7641       | 19             | 48.9         | 47.4           |
|                                          | C4 TGTGCAGTCAGGGTTGCTC          | 7472–7490       | 19             | 53.2         | 57.9           |
|                                          | G4 GCAGTGGGGGGATGTACTG          | 8967–8985       | 19             | 55.4         | 63.2           |
|                                          | T4 TGGATGGTCAGCCCTGCT           | 7504–7522       | 19             | 55.4         | 63.2           |

\(^{\dag}\)References from IMGT/HLA databases [14].

\(^{†}\)Calculation of melting temperature (Tm) is described in Materials and Methods

Underlines, a mismatched base, A, C, G, or T, utilizing binding errors of both purine and pyrimidine.

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Table 3. First designed 36 allele-specific extension primers.

| Names of allele-specific extension primers | Sequence (5'-3') | SNP(s) found in Korean* | Region§ | Length | Tm (°C)† | GC contents (%) | Quality of primer§ | MICB alleles |
|---------------------------------------|-----------------|------------------------|---------|--------|---------|----------------|-------------------|---------------|
|                                       |                 |                        |         |        |         |                |                   |               |
| **MICB 86G**                          | CAGAGCCCCACAGTCTCGG | G                     | 7420–7438 | 19     | 55.4    | 63.2           | ND                |               |
| **MICB 86A**                          | CAGAGCCCCACAGTCTCTCA | —                     | 7420–7438 | 19     | 53.2    | 57.9           | ND                | *023          |
| **MICB 116G**                         | TGTTGCCTGCTCCCAGGATGQG | G                     | 7450–7468 | 19     | 55.4    | 63.2           | ND                |               |
| **MICB 116A**                         | TGTTGCCTGCTCCCAGGATGA | —                     | 7450–7468 | 19     | 53.2    | 57.9           | ND                | *001          |
| **MICB 163C**                         | GGACACTGTGATGTCACQC | C                     | 7497–7515 | 19     | 53.2    | 57.9           | +                 |               |
| **MICB 163T**                         | GGACACTGTGATGTCACTG | —                     | 7497–7515 | 19     | 51.1    | 52.6           | –                 | *024          |
| **MICB 203C**                         | AGAAACGGCCAGGCAAGCC | C                     | 7537–7555 | 19     | 53.2    | 57.9           | ND                |               |
| **MICB 203A**                         | AGAAACGGCCAGGCAAGCA | —                     | 7537–7555 | 19     | 51.1    | 52.6           | ND                | *011          |
| **MICB 223G**                         | AGGAGACGTGGGCGAGAAAG | G                     | 7558–7575 | 18     | 52.6    | 61.1           | +                 |               |
| **MICB 223A**                         | AGGAGACGTGGGCGAGAAAG | A                     | 7557–7575 | 19     | 53.2    | 57.9           | +                 | *004:01       |
| **MICB 238A**                         | GAARATGTCCTGGGAGGCTA | A                     | 7572–7590 | 19     | 51.1    | 47.4           | –                 | *018          |
| **MICB 238G**                         | GAARATGTCCTGGGAGGCTG | G                     | 7572–7590 | 19     | 53.2    | 52.6           | –                 | *002:01       | *019          |
| **MICB 263A**                         | GGACACAGAGACCGAGGA | A                     | 7598–7615 | 18     | 52.6    | 61.1           | ND                |               |
| **MICB 263G**                         | GGACACAGAGACCGAGGG | —                     | 7598–7615 | 18     | 54.9    | 66.7           | ND                | *022          |
| **MICB 314A**                         | GACCTGACTCTATCAAGGA | A                     | 7646–7666 | 21     | 52.4    | 47.6           | ND                |               |
| **MICB 314G**                         | ACCCTGACTCTATCAAGGG | G                     | 7647–7666 | 20     | 51.8    | 50.0           | ND                | *012          |
| **MICB 363C**                         | GATTAGGGTCTGTGAGATC | C                     | 7968–7986 | 19     | 48.9    | 47.4           | +                 |               |
| **MICB 363G**                         | GATTAGGGTCTGTGAGATG | —                     | 7968–7986 | 19     | 48.9    | 47.4           | –                 | *008          |
| **MICB 406G**                         | TCCCGGCTTATTCTACTACG | G                     | 8011–8029 | 19     | 51.1    | 52.6           | +                 |               |
| **MICB 406A**                         | TCCCGGCTTATTCTACAG | —                     | 8011–8029 | 19     | 48.9    | 47.4           | –                 | *002:01       | *018          |
| **MICB 577C**                         | CTGCTGACGAAACTACAGC | C                     | 8181–8201 | 20     | 53.8    | 55.0           | +                 |               |
| **MICB 577T**                         | TGCTGACGAAACTACAGT | T                     | 8182–8201 | 19     | 48.9    | 47.4           | –                 | *009N         |
| **MICB 635C**                         | CCCCATGGTGAATGTCAC | C                     | 8831–8849 | 19     | 53.2    | 57.9           | +                 |               |
| **MICB 635T**                         | CCCCATGGTGAATGTCAT | T                     | 8831–8849 | 19     | 51.1    | 52.6           | –                 | *003          |
| **MICB 643G**                         | GTGAAATGTCAYCTGAGCGG | G                     | 8838–8857 | 20     | 55.9    | 55.0           | ND                |               |
| **MICB 643A**                         | GTGAAATGTCACCTGAGCA | —                     | 8838–8857 | 20     | 53.8    | 55.0           | ND                | *006:015      |

(Continued)
Development of Microarrays for MICB Genotyping Using ASPE

Table 3. (Continued)

| Names of allele-specific extension primers | Sequence (5’-3’) | SNPs found in Korean* | Region‡ | Length | Tm (°C)† | GC contents (%) | Quality of primer§ | MICB alleles |
|-------------------------------------------|------------------|---------------------|--------|--------|----------|-----------------|-----------------|-------------|
|                                            |                  |                     |        |        |          |                 |                 |             |
| *MIB 699G                                 | TTCCAGCTTATCCCCCGG | G                   | 8895–8913 | 19     | 53.2     | 57.9           | +               |             |
| *MIB 699A                                 | TTCCAGCTTATCCCCGA | A                   | 8895–8913 | 19     | 51.1     | 52.6           | –               | *005:03     |
| *MIB 762G                                 | CAACACCGAGTGGGGGG | G                   | 8958–8976 | 19     | 57.6     | 68.4           | +               |             |
| *MIB 762T                                 | CAACACCGAGTGGGGTG | –                   | 8958–8976 | 19     | 55.4     | 63.2           | +               | *005:06     |
| *MIB 836G                                 | GCCAAGGAGAGGAACAG | G                   | 9032–9050 | 19     | 55.4     | 63.2           | ND              |             |
| *MIB 836A                                 | GCCAAGGAGAGGAACAGA | –                   | 9032–9050 | 19     | 53.2     | 57.9           | ND              | *007        |
| *MIB 870C                                 | GGAACACCGGGGATACG | C                   | 9066–9084 | 19     | 53.2     | 57.9           | +               |             |
| *MIB 870T                                 | GGAACACCGGGGATACGT | –                   | 9066–9084 | 19     | 51.1     | 52.6           | +               | *005:01     |
| *MIB 871G                                 | GGAACACCGGGGATACGA | G                   | 9067–9085 | 19     | 53.2     | 57.9           | +               |             |
| *MIB 871T                                 | GGAACACCGGGGATACCA | A                   | 9067–9085 | 19     | 51.1     | 52.6           | +               | *014        |

*-, not present in Korean
‡References from IMGT/HLA databases [14].
§+, successful primer
–, failed primer; ND, not determined in our control panel (Table 1).

Underlines, MICB polymorphic sites.

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(Bioprince; Enzymomics; Daejeon, Korea) and 10 μmol/L of each primer. The reactions were amplified using a Mycycler Thermo Cycler (Bio-rad Inc.; CA, USA) with the following conditions: initial denaturation of 98°C for 20 s; 8 cycles of denaturation at 98°C for 5 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s; followed by 37 cycles of denaturation at 98°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and followed by a final extension at 72°C for 10 min. Then, the reactions were held at 20°C.

**Primer extension on glass slides**

The PCR products were treated with 10× λ exonuclease buffer containing 3.5U λ exonuclease (Fermentas; Burlington, Canada) and 1.75U shrimp alkaline phosphate (Qiagen; Hamburg, Germany). After the PCR products had been cleaved into approximately 50-bp fragments, they were hybridized with a mixture that contained 25× Thermo Sequenase buffer (Enzymomics; Daejeon, Korea), 3.5U Thermo Sequenase (diluted 1:8 in Sequenase dilution buffer; Enzymomics; Daejeon, Korea), and 0.1 nmol/L each of both dATP/dTTP/dGTP and Cy5-dCTP at 60°C for 1 h. To remove non-specific primers, the slides were washed for five minutes with a solution containing 0.1% Alconox and then rinsed twice with distilled water, for 5 min each time.
Table 4. Allele-specific extension primers improved by length and refractory modification.

| Names of allele-specific extension primers | Sequence (5’-3’) | Region‡ | Length | Adjustment of bases | Tm (°C)† | GC contents (%) | Quality of primer |
|-------------------------------------------|------------------|---------|--------|---------------------|---------|-----------------|------------------|
| **Length modification**                   |                  |         |        |                     |         |                 |                  |
| MICB 163T                                 | GGACATCTGGATGGTCAGT | 7497–7515 | 19     | 0                   | 51.1    | 52.6            | –                |
| MICB 163T-1                               | ACATCTGGATGGTCAGT | 7449–7515 | 17     | –2                  | 44.6    | 47.1            | +                |
| MICB 238G                                 | GAARATGCTCTGGGAGCTG | 7572–7590 | 19     | 0                   | 53.2    | 52.6            | –                |
| MICB 238G-1                               | ARATGCTCTGGGAGCTG | 7574–7590 | 17     | –2                  | 49.5    | 52.9            | –                |
| MICB 238G-2                               | ATGTCTCTGGGAGCTG  | 7576–7590 | 15     | –4                  | 44.7    | 60.0            | +                |
| MICB 577T                                 | TGCTGCAGAAACTACAGT | 8181–8201 | 19     | 0                   | 48.9    | 47.4            | –                |
| MICB 577T-1                               | CCTGCAGAAACTACAGT | 8183–8201 | 17     | –2                  | 44.6    | 47.1            | –                |
| MICB 577T-2                               | TGCAAGAAACTACAGT  | 8185–8201 | 15     | –4                  | 36.5    | 40.0            | +                |
| MICB 635T                                 | CCCCCATGGTGAATGTCAT | 8831–8849 | 19     | 0                   | 51.1    | 52.6            | –                |
| MICB 635T-1                               | CCCATGGTGAATGTCAT | 8833–8849 | 17     | –2                  | 44.6    | 47.1            | +                |
| MICB 699A                                 | TTCCAGCTTCTATCCCCGA | 8895–8913 | 19     | 0                   | 51.1    | 52.6            | –                |
| MICB 699A-1                               | CCAGCTTCTATCCCCGA | 8897–8913 | 17     | –2                  | 49.5    | 58.8            | –                |
| MICB 699A-2                               | AGCTTCTATCCCCGA   | 8899–8913 | 15     | –4                  | 41.9    | 53.3            | +                |
| **Refractory modification**               |                  |         |        |                     |         |                 |                  |
| MICB 238A                                 | GAARATGCTCTGGGAGCTA | 7572–7590 | 19     | 0                   | 51.1    | 47.4            | –                |
| MICB 238A-1                               | CAGAARATGCTCTGGGAGCTA | 7570–7590 | 21     | +2                  | 54.4    | 47.6            | –                |
| MICB 238A-2                               | GGCAGAARATGCTCTGGGAGCTA | 7568–7590 | 23     | +4                  | 58.8    | 52.2            | –                |
| MICB 238A-3                               | GGCAGAARATGCTCTGGGAGCTA | 7568–7591 | 24     | +5                  | 59.1    | 50.0            | +                |
| MICB 363G                                 | GATTAGGGTCTGTGAGATG | 7968–7986 | 19     | 0                   | 48.9    | 47.4            | –                |
| MICB 363G-1                               | GAGATTAGGGTCTGTGAGATG | 7966–7986 | 21     | +2                  | 52.4    | 47.6            | –                |
| MICB 363G-2                               | GAGATTAGGGTCTGTGAGATG | 7966–7987 | 22     | +3                  | 54.8    | 50.0            | +                |
| MICB 406A                                 | TCCCGGCATTTCTACTACA | 8011–8029 | 19     | 0                   | 48.9    | 47.4            | –                |
| MICB 406A-1                               | GCTCCCGGCATTTCTACTACA | 8009–8029 | 21     | +2                  | 54.4    | 52.4            | –                |
| MICB 406A-2                               | GCTCCCGGCATTTCTACTACA | 8009–8030 | 22     | +3                  | 54.8    | 50.0            | –                |
| MICB 406A-3                               | GCTCCCGGCATTTCTACTACA | 8009–8031 | 23     | +4                  | 55.3    | 47.8            | +                |

†References from IMGT/HLA databases [14].
‡+, successful primer
§−, failed primer.
Underlines, MICB polymorphic sites.

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The data were obtained using the EasyScan software after scanning with NanoDscan (Nanostorage; Seoul, Korea) and then analyzed for genotyping using Microsoft Excel. The background mean fluorescence intensity (MFI) was subtracted from the MFI of each primer; cut-off values were determined using quality control samples (Fig 1).

**PCR-SBT for MICB**

MICB primers were designed as previously described [16]. PCR was carried out in a reaction volume of 10 μL containing 200 ng genomic DNA, 1× buffer (60 mmol/L Tris-Cl, 15 mmol/L Ammonium Sulfate, 100 mmol/L MgCl2), 200 μmol/L dATP/dGTP/dCTP/dTTP, 0.6 U Taq DNA polymerase (Enzyomics; Daejeon, Korea), bovine serum albumin, Cresol red (in 28% sucrose) and 3.7 μmol/L of each primer. The cycling conditions were as follows: 95°C for 5 min; then 19 cycles of 94°C for 45 s, 65°C for 45 s (a reduction of −0.5°C per cycle), and 72°C for 2 min; then 13 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 2 min; and finally, 72°C for 10 min. Then, the reactions were held at 20°C.

The MICB exon 6 primers were designed to differentiate MICB*005:02 allele from MICB*010 allele in exon 6, with the forward primer as 5’-GGGCAACTGAAGAGAGAAAAG-3’ and the reverse primer as 5’-CAGGAGCAGTCGTGAGTTTG-3’. The PCR conditions were as follows: an initial denaturation of 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. Then the reactions were held at 20°C.

Purified PCR products were sequenced using an ABI 377 DNA sequencer; the MICB primers for PCR amplification were also used as the sequencing primers. The sequencing data were analyzed using the ABI Factura software and the ABI Sequence Navigator program (PE Biosystems; Mississauga, Canada).
Calculation of melting temperature and statistical analysis

The melting temperature (Tm) for sequences longer than 13 bases was calculated as follows:

\[
Tm = 64.9 + \frac{41 \times (yG + zC - 16.4)}{wA + xT + yG + zC}
\]

W, x, y, and z indicate the number of adenine, thymine, guanine and cytosine (A, T, G, and C nucleotides), respectively, in the sequence.

The Tm and GC content in primers from the successful group of allele-specific extension primers were compared to those from the failed group using the unpaired Student’s t-test. We corrected for statistical analyses calculated from five or less observed scores using Fisher’s exact test.

Results

Control extension primers for quality control

For quality control, 20 extension primers were designed against sequences in MICB exon 2, 3, 4, and 5. The primers were 19 bp long, with a Tm of 53.0 ± 3.0°C, and a GC content of 57.4 ± 7.4% (Table 2). The MFI value of the N0 primers was higher than the maximum value (65,535 MFI) of N0 primers measured by the EasyScan-1 scanning machine, and the MFI value for the N4 primers was >30,000. By contrast, the N1 primer group produced a very weak signal, whereas N2 and N3 displayed refractory extension (Fig 1). As the N0 and N4 primers showed strong positive signals, they were used as positive controls, and the primers in the other groups were used as negative controls. Based on these results, we set very strict and reliable cut-off values for MFI: an MFI >30,000 was considered as a positive signal, and an MFI <10,000 was considered a negative signal. Using these cut-offs, it was possible to select high quality allele-specific extension primers.

Optimization of allele-specific extension primers

The ability to discriminate MICA alleles from MICB alleles was verified using primer location and a cross over test. Primers for amplification of exons 2–5 of MICB alleles did not exist in the MICA gene (Gene ID: 100507436). The PCR products of MICB exons 2–5 did not showed any positive signals on the MICA allele-specific extension primers of microarray for MICA allele genotyping [17].

Eighteen samples including 13 MICB alleles previously identified in Koreans and other populations were selected as the quality control panel for ASPE on microarrays. We defined MICB alleles of 163 samples in the UCLA International KIR and MICA Exchange Report program. Among them, six samples including three MICB alleles of MICB*005:01, *005:06 and *024 alleles which did not find in Korean population were added in Table 1. MICB alleles of the other 157 samples were also found in Korean population. A validation of the amplification reaction has been performed for the alleles (MICB*002:01, *003, *004:01, *005:01, *005:02/010, *005:03, *005:06, *008, *009N, *014, *018, *019, and *024 alleles) included in the quality control panel. To identify the 41 known MICB alleles, 36 allele-specific extension primers were designed with a length of 18 to 21 bases. Among these primers, the specificity and sensitivity of only 22 primers could be determined by the quality control panel used in this study (Table 3). Of these primers, 14 showed excellent sensitivity and specificity with a Tm of 52.9 ± 2.1°C and a GC content of 57.2 ± 5.1%. The eight failed primers had a Tm of 50.6 ± 1.5°C and a GC content of 50.0 ± 2.8%. The Tm and GC content were significantly different between the successful extension primer group and the failed primer group (P < 0.02 for each category).
Of the eight failed primers, five were optimized by shortening the primer at the 5' end to eliminate false positive signals, and the other three were optimized by correcting mismatches that were refractory to extension (Fig 2; Table 4). All the MICB'002:01, '003, '005:02/'010, '005:03, '008, '009N, '018, and '024 alleles present in our panel showed perfect matches by 22 allele-specific extension primers validated with the quality control panel after optimal modification of primers (Tables 3 and 4). Although alleles showing mismatches did not exist in our panel, rare alleles still need to be further defined. The MICB'005:02/'010 allele was verified by negative signals of 14 allele-specific primers matching the other 12 MICB alleles above. We speculate three were allele and genotype ambiguities not resolvable by ASPE in the above condition such as MICB'004:01/'020/'025/'026/'028, '005:06/'005:07/'005:08/'010/'021N/'027/'030 and '002:01/'029.

**Genotyping of MICB alleles**

The ASPE on microarrays were evaluated using the arrays that genotyped the 18 SNPs in the MICB alleles. The samples of known MICB alleles from about 200 Koreans and six individuals of other populations were analyzed for genotyping. Scatter plots were resulted in numerical presentation about one clustering of three genotypes. The similar values between the signals from the normal allele-specific reactions were divided into three districts depending on the genotype; 20 SNPs were over 30,000 MFI (Fig 3). Scatter plots showed that the allele-specific
Reactions could be divided into three groups (223AA/AG/GG, 238AA/AG/GG, and 406AA/AG/GG). Another 8 SNP regions had one heterozygote and one homozygote (163CT/CC, 363CG/GG, 577CT/TT, 635CT/TT, 699AG/GG, 762GT/GG, 870CT/CC, and 871AG/GG). The other seven SNPs existed in the homozygous state (86GG, 116GG, 203CC, 263AA, 314AA, 643GG, and 836GG).

Analysis of MICB alleles using the 22 allele-specific extension primers resulted in 27 different combinations of SNPs (Table 5). The most common genotype was homozygous for MICB/C3005:C302/C3010 (n = 56), followed by MICB/C3005:C302/C3010/C3004:C301 (n = 32), MICB/C3005:C302/C3010/C3005:C303 (n = 20), MICB/C3005:C302/C3010/C3008 (n = 15), MICB/C3005:C302/C3010/C3002:C301 (n = 10), MICB/C3005:C302/C3010/C3005:C301 (n = 10), MICB/C3005:C302/C3010/C3005:C301 (n = 8), and MICB/C3005:C302/C3010/C3002:C301 (n = 8). The other 9 combinations were seen in only one sample each (MICB/C3002:C3018, MICB/C3002:C3019, MICB/C3003/C3005, MICB/C3004:C3014, MICB/C3005:C300/C3005:C3014, MICB/C3005:C3001, MICB/C3005:C3001 and MICB/C3001:C3024). Because this MICB alleles' genotyping on microarrays with ASPE can discriminate the 18 SNPs among 28 SNPs known in MICB gene (Table 5), the other 10 SNPs of MICB did not detect and not discriminate all alleles, existed many ambiguous allele combinations. These SNPs are present only in very rare alleles which have not been reported in published papers and are not found in 163 samples of the UCLA International Exchange Report program. Therefore, it may have some limitations for application compared with direct...
Table 5. *MICB* alleles defined by ASPE on microarrays.

| Allele 1 | Allele 2 | n = 206 | A/G | A/G | C/T | A/C | A/G | A/G | C/G | A/G | C/T | C/T | A/G |
|----------|----------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| *005:02/ *010 | *005:02/ *010 | 56 | GG | GG | CC | CC | GG | AA | AA | AA | CC | GG | CC | CC | GG | GG | GG | GG | CC | GG |
| *005:02/ *010 | *004:01 | 32 | GG | GG | CC | CC | AG | AA | AA | AA | CC | GG | CC | CC | GG | GG | GG | GG | CC | GG |
| *005:02/ *010 | *002:01 | 30 | GG | GG | CC | CC | GG | AG | AA | AA | CC | AG | CC | CC | GG | GG | GG | GG | CC | GG |
| *005:02/ *010 | *005:03 | 16 | GG | GG | CC | CC | GG | AA | AA | AA | CC | AG | CC | CC | GG | AG | GG | GG | CC | GG |
| *005:02/ *010 | *008 | 15 | GG | GG | CC | CC | GG | AG | AA | AA | CC | AG | CC | CC | GG | GG | GG | GG | CC | GG |
| *005:02/ *010 | *009N | 8 | GG | GG | CC | CC | GG | AA | AA | AA | CC | GG | CT | CT | GG | GG | GG | GG | CC | GG |
| *005:02/ *010 | *003 | 8 | GG | GG | CC | CC | GG | AA | AA | AA | CC | CC | CT | GG | GG | GG | GG | CC | GG |
| *002:01 | *014 | 6 | GG | GG | CC | CC | GG | GG | AA | AA | CC | AA | CC | CC | GG | GG | GG | GG | CC | AG |
| *002:01 | *008 | 4 | GG | GG | CC | CC | GG | GG | AA | AA | CC | AA | CC | CC | GG | GG | GG | GG | CC | GG |
| *004:01 | *004:01 | 4 | GG | GG | CC | CC | AA | AA | AA | AA | CC | GG | CC | CC | GG | GG | GG | GG | CC | GG |
| *002:01 | *004:01 | 4 | GG | GG | CC | CC | AG | AG | AA | AA | CC | AG | CC | CC | GG | GG | GG | GG | CC | GG |
| *002:01 | *005:03 | 2 | GG | GG | CC | CC | GG | AG | AA | AA | CC | AG | CC | CC | GG | AG | GG | GG | CC | GG |
| *003 | *008 | 2 | GG | GG | CC | CC | GG | AG | AA | AA | CC | AG | CC | CC | GG | AG | GG | GG | CC | GG |
| *005:03 | *009N | 2 | GG | GG | CC | CC | GG | AA | AA | AA | CC | GG | CT | CT | GG | AG | GG | GG | CC | GG |
| *002:01 | *003 | 2 | GG | GG | CC | CC | GG | AG | AA | AA | CC | AG | CC | CT | GG | GG | GG | GG | CC | GG |
| *005:02/ *010 | *005:01 | 2 | GG | GG | CC | CC | GG | AA | AA | AA | CC | CC | CC | GG | GG | GT | GG | CT | GG |
| *005:02/ *010 | *005:06 | 2 | GG | GG | CC | CC | GG | AA | AA | AA | CC | CC | CC | GG | GT | GG | CT | GG |
| *002:01 | *002:01 | 2 | GG | GG | CC | CC | GG | GG | AA | AA | CC | AA | CC | CC | GG | GG | CC | CC | GG | GG |
| *002:01 | *018 | 1 | GG | GG | CC | CC | GG | AG | AA | AA | CC | AA | CC | CC | GG | GG | GG | GG | CC | GG |
| *002:01 | *019 | 1 | GG | GG | CC | CC | GG | AA | AA | AA | CC | AG | CC | CC | GG | GG | GG | GG | CC | GG |
| *003 | *005:03 | 1 | GG | GG | CC | CC | GG | AA | AA | AA | CC | GG | CC | CT | GG | AG | GG | GG | CC | GG |
| *004:01 | *014 | 1 | GG | GG | CC | CC | AG | AG | AA | AA | CC | AG | CC | CC | GG | GG | GG | GG | CC | AG |
| *005:02/ *010 | *014 | 1 | GG | GG | CC | CC | AG | AG | AA | AA | CC | AG | CC | CC | GG | GG | GG | GG | CC | AG |
| *003 | *004:01 | 1 | GG | GG | CC | CC | GA | AA | AA | AA | CC | GG | CC | CT | GG | GG | CC | GD | CC | GG |
| *003 | *009N | 1 | GG | GG | CC | CC | GG | AA | AA | AA | CC | GG | CT | TT | GG | GG | CC | CC | GG |
| *005:03 | *005:01 | 1 | GG | GG | CC | CC | GG | AA | AA | AA | CC | GG | CC | CC | GG | AG | GG | GG | CT | GG |
| *004:01 | *024 | 1 | GG | GG | CT | CC | AA | AA | AA | AA | CC | CC | CC | GG | GG | GG | GG | CC | GG |

*Bold letters showed three genotypes or one heterozygote and one homozygote.*

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Genotyping by ASPE on microarrays was fully concordant with *MICB* genotyping using PCR-SBT in the sample-to-sample comparison (Table 6). *MICB*’002:01 and *004:01 alleles, which were determined using ASPE on microarrays, were identified as *MICB*’002:01:01 and *MICB*’004:01:01 alleles by PCR-SBT. Our SBT method could discriminate *MICB*’010 allele.
from *MICB*’005:02 allele because these two alleles are different in exon 6. We could identify only *MICB*’005:02 allele in Korean population using PCR-SBT (n = 99). However, we showed *MICB*’005:02/*010 allele because the primer sets used to amplify templates for ASPE on micro-arrays were not designed to differentiate *MICB*’005:02 allele from *MICB*’010 allele in exon 6. *MICB*’018 and ’019 alleles, which have not previously been reported in Koreans, were found in this study.

**Discussion**

The ASPE method depends for identifying alleles on the sequence-specific extension of immobilized allele-specific extension primers that differ at the 3’ ends of their SNPs [11]. However, certain mismatches are not refractory to extension. The systemic characteristics and optimization of the ASPE procedure have been shown to be influenced by annealing temperature, template concentration, and Mg2+ concentration [18]. Of the extension primers examined in this study, which were designed to have a length of 18 to 21 bases, 14 showed excellent sensitivity and specificity in the control panel (Table 3).

Allele-specific PCR with *Taq* polymerase offers the greatest template discrimination (40- to 100-fold) against mismatches to thymine, guanine, or cytosine (T, G, or C) at the 3’ end of a primer, but not against mismatch to adenine (A) [19]. To improve the accuracy, we used the thermostable Thermo Sequenase which was engineered to catalyze the incorporation of dNTPs with higher efficiency than other DNA polymerases on SBE-based microarray [20]. For primers with cytosine (C) as the 3’ nucleotide, Thermo Sequenase was highly specific for template complementarity to this base (Table 4). By contrast, primers with thymine (T) at the 3’ end were less efficiently amplified regardless of the corresponding nucleotide on the template strand. These results are directly applicable to the design of primers for SNP detection and have made it possible to develop a general procedure to increase the specificity of extension primers by specifying an additional variable nucleotide at the 3’ end of the extension primer.

Different approaches have been proposed to improve the specificity of ASPE. As the specificity of primer-directed extension is not sufficient for quantitative SNP analysis, artificial mismatched bases have been introduced into the 3’ end regions of the specific primers as a way of improving the switching characteristics of the primer extension reactions. The best position in the primer for such artificial mismatched bases is the third position from the 3’ end of the
primer [21]. In our control extension primers, mismatched control extension primers containing errors in the 2nd and 3rd bases from the 3' end gave a completely negative signal, whereas primers containing an error in the 1st base from the 3' end gave a weak signal (Fig 1). The specificity of extension primers targeting SNP region was also increased by the modification for refractory extension (Fig 2; Table 4). For further improvement of primer quality, apyrase, a nucleotide-degrading enzyme to the extension reaction, should be introduced to DNA microarrays [22, 23]. We did not use apyrase in our assay and the quality of primers was improved by optimal modifications instead. We retested the reactions if the MFI value was between 10,000 and 30,000. When the same values appeared over three trials, the results were confirmed using other methods such as PCR-SBT (data not shown).

Although we designed 36 primers to detect almost all of the known 41 MICB alleles, only 22 primers could be defined or improved using the control panel in this study. Therefore, we could not define the other rare alleles and it is necessary to confirm the quality of the 14 remaining primers using samples from other populations. In Korean population, the MICB alleles detected by the genotyping microarray were fully in concordance with those detected by PCR-SBT and we compared the MICB population allele frequency (Korean population) with the test sample frequency in Table 6 (2n = 400). Two of the alleles found in this study, MICB*018 and '019 alleles, had not been previously found in Koreans [16]. Therefore, it was possible to identify 13 MICB alleles in this study using verified allele-specific extension primers. Our microarray did not include SNP in exon 6 because these SNPs are present only in very rare alleles which have been not reported in population data of published papers and are not present even in 163 samples of the UCLA International Exchange Report program. Although we designed primers to detect SNPs in exon 2 to 5 of MICB, new alleles have recently been defined by SNPs in exon 6 [24]. MICB*010 and '005:02 alleles could not be discriminated by ASPE on microarrays because the primers had not been designed to differentiate MICB*005:02 allele from MICB*010 allele in exon 6.

The 13 MICB alleles identified by this method were MICB*002:01, *003, *004:01, *005:01, *005:02/'010, *005:03, *005:06, *008, *009N, *014, *018, *019 and *024 (Table 6). However, the rare 28 MICB alleles could not be identified; MICB*001, *005:04, *005:05, *005:07, *005:08, *006, *007, *011, *012, *015, *016, *017, *020, *021N, *022, *023, *025, *026, *027, *028, *029 and *030. Therefore, some MICB alleles can not discriminate and may result in error data.

If the frequency of the minor allele is greater than 1%, such variants are called polymorphisms. These 13 MICB alleles were found more than 1% in reported ethnic populations including Welsh, Spanish, and Chinese and the other alleles are present less than 1% only in particular populations [5, 10, 16, 24–26]. Therefore, this method may be useful for disease association studies in various populations, even though it can not detect rare alleles.

We had previously developed a typing method of the SNPs of cytokine genes using ASPE on a fluorescence bead array [27]. According to this study and the bead array study, we showed that allele typing could not only be possible on liquid phase but also on solid phase. Although Sanger and next generation sequencing is readily available in labs and new alleles can be detected, this DNA chip-based assay allows for a multiplex assay to detect several mutations in addition to a being relatively rapid and cost-effective means of detecting defined mutations for genetic diagnosis.

Conclusions
We have established a system for genotyping MICB alleles using ASPE on microarrays. Available operation of control and allele-specific extension primers was representatively confirmed by schematic patterns of successful allele-specific extension primers that could discriminate 13
MICB alleles (S1 and S2 Figs). In conclusion, our method for genotyping MICB alleles using ASPE on microarrays could be applicable for large-scale SNP typing studies of population and disease associations.

Supporting Information

S1 Fig. Schematic patterns of 22 allele-specific primers that could discriminate 13 MICB alleles.

S2 Fig. Available operation of successful allele-specific primers representatively confirmed by genotyping of 10 MICB alleles, using ASPE on microarrays.

Author Contributions

Conceived and designed the experiments: ICB TGK. Performed the experiments: ICB. Analyzed the data: ICB. Contributed reagents/materials/analysis tools: ICB EJC. Wrote the paper: ICB JPJ.

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