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Associations of *MICB* with cervical cancer in north-eastern Thais: identification of major histocompatibility complex class I chain-related gene B motifs influencing natural killer cell activation

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Summary

The expression of *MICB*, a member of the major histocompatibility complex class I chain-related gene B family, is induced in response to cellular stress. It is one of the ligands to the NKG2D receptor. *MICB* is polymorphic, but the distribution of *MICB* polymorphism in north-eastern Thais and their potential associations with cancer have not yet been elucidated. In this study, polymerase chain reaction–sequence-specific primers were developed to identify 15 *MICB* alleles and one group of alleles. We performed *MICB* typing in 100 healthy north-eastern Thai females (NETF) and 99 cervical cancer patients to evaluate the association of *MICB* polymorphisms and the risk of developing cervical cancer. Eight and nine alleles were detected in the NETF and cervical cancer respectively. *MICB*00502* was associated negatively with a corrected *P*-value of 0·0009, suggesting the existence of a protective allele in cervical cancer. Amino acid substitutions carried by this allele were investigated for their potential involvement in natural killer (NK) cell activation. Although lysine at amino acid position 80 (Lys80) and aspartic acid at position 136 (Asp136) were associated negatively with cervical cancer, only MICB carrying Asp136 could induce NK cell killing more efficiently than MICB-Lys80 when the NK cells were blocked by anti-NKG2D. This result suggested that aspartic acid at position 136 may affect NKG2D binding, leading to different degrees of immune cell activation.

Keywords: activation, cancer, genetic, non-classical MHC

Introduction

Cervical cancer (CXCA) is currently the third most common type of cancer among women worldwide [1] and the second most common cancer among women in the developing world [2]. Even though screening programmes based on the Papanicolaou (Pap) smear and pelvic examination have led to a steep decline in incidence and deaths from CXCA, several hundred thousands of new cases are diagnosed each year, predominantly in both developing and industrialized countries. In Thailand, the incidence of CXCA is about 23·4 cases per 100 000 women [3]; however, the diagnosis is made usually in advanced stages. Infection with human papilloma virus (HPV) types 16 or 18 is the major cause of cervical neoplasia [4]. Although a high proportion of CXCA harbour HPV genomes, only a small number of women infected with high-risk papilloma viruses develop cervical tumours, suggesting that other environmental, genetic factors and/or immune surveillance contribute to cervical carcinogenesis.

Down-regulation of major histocompatibility complex (MHC) class I and induction of MHC class I chain-related

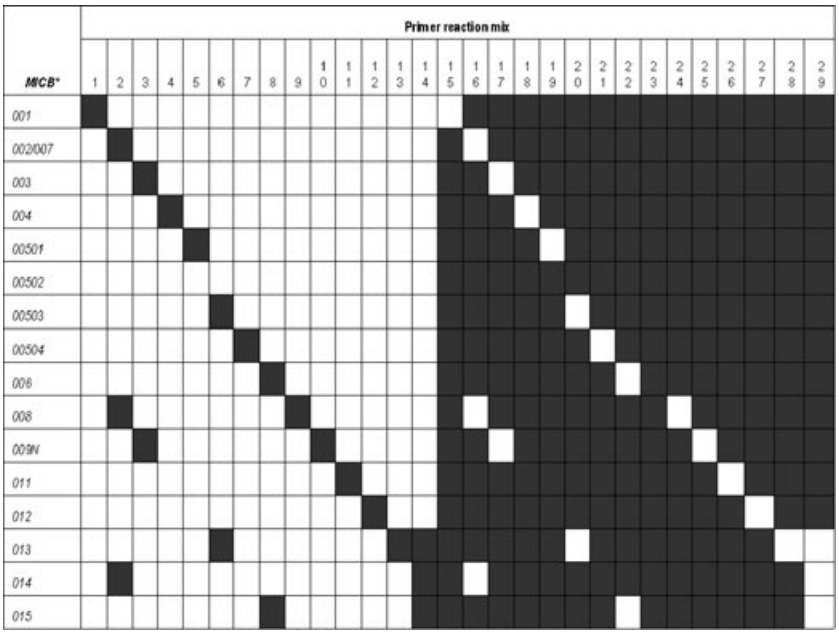

Fig. 1. Interpretation of major histocompatibility complex class I chain-related gene B (MICB) alleles based on polymerase chain reaction (PCR)–sequence-specific primers (PCR) of 29 PCR reaction mixes. *MICB* alleles are listed on the left. The numbers of PCR reaction mixes are shown on the top. Black boxes represent positive PCR reactions with the primer mixes, as indicated at the top of each column. Primer mixes numbers 15–29 were designed for substitutions at allele-specific sites in order to identify a sample carrying homozygous *MICB* allele.

molecules, MIC, have been observed in various tumour types. MIC proteins, mainly MHC class I chain-related gene A (MICA) and MHC class I chain-related gene B (MICB), are induced only in response to cellular stress on limited cell types, restricted essentially to epithelial-derived tumours [5,6]. MIC can activate killing activity of natural killer (NK) cells against cancerous cells via the NKG2D and DAP10 or DAP12 complex [7,8] expressed on the cell surface of many immune cells, e.g. NK cells, some cytotoxic CD8 αβT cells, γδT cells, NK T cells, macrophages and a small subset of CD4 αβT cells [7,9–11]. Thus, NKG2D-ligand recognition plays an important role in both innate and adaptive immune systems.

MICA and *MICB* are polymorphic [12,13]; however, *MICB* polymorphism seems to be lower than that of MICA. To date, at least 57 alleles of *MICA* were identified whereas 18 *MICB* alleles were reported [14]. The extent of *MICB* polymorphism and its distribution in populations remain under investigation. Consequently, the potential association of *MICB* to diseases has not been evaluated. In this study, we have described the development of a polymerase chain reaction (PCR) amplification method using sequence-specific primers (SSP) discriminating *MICB* alleles. This method was employed to type *MICB* of healthy north-eastern Thai females (NETF) as well as CXCA patients in order to evaluate the association of the *MICB* alleles with this disease. In addition, potentially functional MICB motifs identified by this association study were tested for their differential binding to NKG2D and NK cell-killing activities.

Materials and methods

Study subjects

The subjects were divided into two groups, CXCA and healthy control subjects. Peripheral blood mononuclear cells collected from citrate phosphate dextrose or ethylenediamine tetraacetic acid blood were used to prepare genomic DNA. The project was approved by the Khon Kaen University Ethical Committee (HE430205) and informed consents were obtained from all subjects participating in the project. Ninety-nine CXCA cases were recruited from the tumour clinic, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University by gynaecological clinicians. All patients were north-eastern Thais and diagnosed histologically. The CXCA samples were classified into different stages following the International Federation of Gynecology and Obstetrics (FIGO system). These samples were categorized into two groups – low grade (stages I and II = 43 cases) and high grade (stages III and IV = 57 cases). In addition, 87% of these cases were positive with HPV (high-risk types: 16, 18 and 33 were 91·2% and low-risk types were 8·8%). Healthy NETF were screened by interview with negative Pap smear. One hundred unrelated female subjects whose families are Thai and have lived in the north-eastern part of Thailand for at least two generations were included as a healthy control group.

Oligonucleotide primers

In this study, we have developed a PCR-based typing system using SSP to identify *MICB* alleles. PCR reaction mixtures composed of 29 primer pairs were used for amplification of 15 *MICB* alleles and one group of alleles (Fig. 1) which have been identified previously [15–21]. The first set of 14 SSP pairs (reaction mixes numbers 1–14) were designed for specific amplifications of the 15 *MICB* alleles and one group of alleles, whereas the remaining primer set was included to identify homozygous alleles. Primers recognizing conserved homologous sequences in the human

growth hormone (hGH) gene were used as internal control primers.

Primer sequences and locations relative to the *MICB* gene are given in Table 1. DNA samples extracted from International Histocompatibility Workshop cell lines with known *MICB* alleles, kindly provided by Dr Campbell Witt, Royal Perth Hospital, Western Australia, were used as positive controls to validate specific amplifications of all designed primers.

Amplification of the *MICB* gene using PCR–SSP

PCR conditions for all designed primers were optimized with their corresponding reference DNA samples. To confirm DNA amplification, each primer mix contained internal control primers which amplified the 439 base pairs (bp) product of the hGH gene. The condition initially followed the standard condition procedure of the 12th International Workshop for human leucocyte antigen typing based on PCR–SSP, with modifications. The final volume of 13 μl of PCR containing 100–200 ng of DNA, 1× PCR buffer, 2·0 mM $MgCl_2$, 0·2 mM 2′-deoxynucleosides 5′-triphosphate mix, 0·5–1·0 μM of specific primers, 0·1 μM of internal control primers and 0·5 units *Taq* DNA polymerase [25]. PCR amplification was carried out using the PTC 200 thermocycler (MJ Research, Oldendorf, Germany). The cycling condition was performed with one cycle of denaturation at 96°C for 2 min and followed subsequently with three different cycling steps: five cycles of 96°C 30 s, 65°C 60 s, 72°C 40 s; 21 cycles of 96°C 30 s, 58°C 60 s, 72°C 40 s; four cycles of 96°C 30 s, 55°C 75 s, 72°C 120 s and finally with one cycle of extension step at 72°C for 10 min. MICB allele-specific PCR products were finally investigated by agarose gel electrophoresis. Successful PCR amplification must be found with a 439 bp product of the hGH internal control primers.

Molecular genetic techniques and construction of plasmids

Genomic DNA of *MICB*014* was obtained by PCR. Forward PCR primer was synthesized corresponding to the *MICB* gene (exon 2–exon 6, 4·2 Kb) by designing the 5′ end of the primers containing a four-nucleotide sequence (CACC) and the signal sequence of *MICB* (PB112-TOPO: CACCAT GGGGCTGGGCCGGGTCCTGCTGTTTCTGGCCGTCGC CCTCCCTTTTGCACCCCCGGCAGCCGCCGCTGAGCC CCACAGTCTTCGT) and the 3′ end of primer containing stop codon (PB11-CYTB-TOPO: GGCGCCCTCAGTGGA (A/G)CCAGTGGAC). The 4·2 Kb PCR product was inserted into the pcDNA 3·1 TOPO vector using the pcDNA 3·1 Directional TOPO Expression Kit (Invitrogen, Carlsbad, CA, USA). The plasmid sequence was validated by sequencing. Then, the codon for Lys80 (AAG > GAG) in α1 and Asp136 (GAT > AAT) in α2 of *MICB*014* (Table 2) were mutated by the site-directed mutagenesis technique. The primers used for mutagenesis were:

E80K forward (5′-CCTGGGAGCTAAGACCTGGGAC-3′), reverse (5′-GTCCCAGGTCTTAGCTCCCAGG-3′); and N136D forward (5′-CATTTCTACTACGATGGGGAGCTC-3′), and reverse (5′-GAGCTCCCCATCGTAGAAATG-3′).

The PCR control reaction with no primer and experiment reaction were performed at the same time. After PCR products were amplified, PCR reactions (both experiment and control reactions) were treated with *DpnI* (Promega, Madison, WI, USA). Then, both of the treated PCR reactions were transformed into competent cells (TOP10 *Esherichia coli*). Colonies were selected by amiplicilin (Sigma-Aldrich, St. Louis, MO, USA). The experimental reaction was accepted when the control reaction had no grown colony on the plate. The resulting plasmid DNAs carrying Lys80 and Asp136 of *MICB* were validated by sequencing to confirm that no inappropriate mutations had occurred.

Major histocompatibility complex class I chain-related molecules stably transfected cells

The C1R cells were transfected with *MICB*014*, mutated *MICB*014*-Lys80 and *MICB*014*-Asp136 to produce stable transfectants by the electroporation method (250 volt and 950 μF) using Genepulser (Biorad, Foster City, CA, USA). Populations of cells expressing MICB were fluorescence activated cell sorter (FACS) sorted and maintained in complete RPMI-1640 with 1 mg/ml G418.

Flow cytometry analysis of transfectants

For flow cytometry analysis, 10^5 cells were preincubated in PBS containing 1% bovine serum albumin, 0·1% sodium azide (Sigma-Aldrich). Cells were stained with different concentrations (20–50 μg/ml) of NKG2D-immunoglobulin (Ig) fusion protein [26] (kindly provided by Dr Hugh T. Reyburn, University of Cambridge, Cambridge, UK) followed, after washing, by phycoerythrin-labelled $F(ab')_2$ fragments of goat anti-human Ig (Beckman Coulter, Fullerton, CA, USA). Staining with mouse anti-MICA/B monoclonal antibodies (mAbs) (1D10, a kind gift of Dr Andrew Brooks, University of Melbourne, Melbourne, Australia) was visualized with phycoerythrin-labelled $F(ab')_2$ fragments of goat anti-mouse Ig (Dako, Carpinteria, CA, USA). Samples were analysed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Natural killer cell isolation and propagation

Primary polyclonal NK cells were prepared from healthy adult donors, as described previously [27]. Pure NK cell populations (> 95% $CD3^-$, $CD56^+$) were obtained from

Table 1. The locations and sequences of oligonucleotide primers used for major histocompatibility complex class I chain-related gene B (MICB) typing by polymerase chain reaction (PCR)–sequence-specific primers (PCR).

Mix no.	Names and primer sequence	Location	Specificity
1	7473A*: GGT GCT GTC CCA GGA TGA	7456–7473	*MICB * 001*
	8224RG*: ACC CCG GAT TTC AGA TAT CG	8224–8205	
2	MICB3F: CAC TTG GGT GGA AAG GTG ATG	7895–7915	*MICB * 002, 007, 008, 014*
	P14R*: GAG GAA GAG CTC CCC ATT	8051–8034	
3	MICB4F: CAG CCC TGT TCC CTG CAT	8765–8782	*MICB * 003, 009N*
	P18R: CTG AGA CCT CGC TGC AGA	8871–8854	
4	MICB2F: GAG GTC GGG ACA GCA GAC	7341–7358	*MICB * 004*
	A104R: CTC AGC TCC CAG GAC ATT	7597–7580	
5	C9089T: GGA ACA CAG CGG GAA TCA T	9071–9089	*MICB * 00501*
	T9236T: CAT ATG GAA AGT CTG TCC GT	9255–9236	
6	A022*: TCC AGC TTC TAT CCC CGA	8901–8918	*MICB * 00503, 013*
	MICB4R: ACA GCC GTC CCT GCT GTT	9171–9154	
7	A023C*: AAT GGA ACC TAC CAG ACG	9000–9017	*MICB * 00504*
	MICB4R: ACA GCC GTC CCT GCT GTT	9171–9154	
8	A006K*: TGA ATG TCA CCT GCA GCA	8845–8862	*MICB * 006, 015*
	MICB4R: ACA GCC GTC CCT GCT GTT	9171–9154	
9	A106: ATT AGG GTC TGT GAG ATG	7974–7991	*MICB * 008*
	MICB3R: GAA TTG CGG GAA CAG TAG AGC	8281–8261	
10	MICB3F: CAC TTG GGT GGA AAG GTG ATG	7895–7915	*MICB * 009N*
	A107NR: CCC GGA TTT CAG ATA TCA	8222–8205	
11	MICB2F: GAG GTC GGG ACA GCA GAC	7341–7358	*MICB * 011*
	A-005R: CTG CCC ACT GTC CCT GGT	7577–7560	
12	A7671G*: ACC CTG ACT CAT ATC AAG GG	7652–7671	*MICB * 012*
	MICB3R: GAA TTG CGG GAA CAG TAG AGC	8281–8261	
13	A022*: TCC AGC TTC TAT CCC CGA	8901–8918	*MICB * 013*
	A022_301VR: GGG CAC AGG GTG AGT GCT	9107–9090	
14	533G‡: CAC ACT ATC GCG CTA TGC AG	8161–8180	*MICB * 014, 013, 015*
	A022_301VR: GGG CAC AGG GTG AGT GCT	9107–9090	
15	7473G*: GGT GCT GTC CCA GGA TGG	7456–7473	*All but not MICB * 001*
	8224RG*: ACC CCG GAT TTC AGA TAT CG	8224–8205	
16	MICB3Fn: AGG AAT AGG GTC AGG GAG G	7790–7808	*All but not MICB * 002, 007, 008, 014*
	PB3R*: GAG GAA GAG CTC CCC ATC	8051–8034	
17	P17†: CCC CAT GGT GAA TGT CAC	8837–8854	*All but not MICB * 003, 009N*
	MICB4R: ACA GCC GTC CCT GCT GTT	9171–9154	
18	MICB2Fn: TTT CCT GCC TCC TCA GGG AG	7324–7343	*All but not MICB * 004*
	7597RC: CTC AGC TCC CAG GAC ATC	7597–7580	
19	MICB4Fn: ACA GAA GGT CTG GGA TCT GT	8709–8728	*All but not MICB * 00501*
	9105RG: GGC ACA GGG TGA GTG CCG	9106–9089	
20	8918G*: TCC AGC TTC TAT CCC CGG	8901–8918	*All but not MICB * 00503, 013*
	MICB4R: ACA GCC GTC CCT GCT GTT	9171–9154	
21	9017C*: AAT GGA ACC TAC CAG ACC	9000–9017	*All but not MICB * 00504*
	MICB4R: ACA GCC GTC CCT GCT GTT	9171–9154	
22	8862G*: TGA ATG TCA CCT GCA GCG	8845–8862	*All but not MICB * 006, 015*
	MICB4R: ACA GCC GTC CCT GCT GTT	9171–9154	
23	9055G: CCA AGG AGA GGA GCA GAG	9038–9055	*All but not MICB * 007*
	MICB4R: ACA GCC GTC CCT GCT GTT	9171–9154	
24	7991C: ATT AGG GTC TGT GAG ATC	7974–7991	*All but not MICB * 008*
	MICB3R: GAA TTG CGG GAA CAG TAG AGC	8281–8261	
25	MICB3F: CAC TTG GGT GGA AAG GTG ATG	7895–7915	*All but not MICB * 009N*
	8224RG: ACC CCG GAT TTC AGA TAT CG	8224–8205	
26	7560C: GAA ACG CAG GGC AAA GCC	7543–7560	*All but not MICB * 011*
	8224RG: ACC CCG GAT TTC AGA TAT CG	8224–8205	
27	MICB2F: GAG GTC GGG ACA GCA GAC	7341–7358	*All but not MICB * 012*
	7671RT: TCT CAC CTC CTT TCT GGT	7688–7671	
28	8918G*: TCC AGC TTC TAT CCC CGG	8901–8918	*All but not MICB * 013*
	9107RC: GGG CAC AGG GTG AGT GCC	9107–9090	
29	533G‡: CAC ACT ATC GCG CTA TGC AG	8161–8180	*All but not MICB * 013, 014, 015*
	9107RC: GGG CAC AGG GTG AGT GCC	9107–9090	

Underlined primer names: primer sequences designed in this study. *The same or modified primer sequences from the study of of Ahmad *et al.* [23]; †Modified primer sequences from the study of of Gonzalez *et al.* [24]; ‡Primer sequences used by the study of Collins *et al.* [28]. Location reference from MICB genomic sequence (Accession number: U65416).

Table 2. Allelic amino acids of major histocompatibility complex class I chain-related gene B (MICB).

	Amino acid position					
MICB	75	80	121	136	212	291
002	D	E	I	N	T	G
003	D	K	I	D	I	G
004	N	K	I	D	T	G
00501/00502/00503	D	K	I	D	T	G
008	D	E	M	N	T	G
013	D	K	I	D	T	S
014	D	E	I	N	T	S

negative selection (Miltenyi Biotec, Gladbach, Germany) and were monitored periodically for the presence of T cells. All the NK cells expressed the activating receptors, NKG2D; mAbs to CD3 and CD56 were purchased from BD Pharmingen (San Diego, CA, USA) and mAb to NKG2D was purchased from R&D Systems (Minneapolis, MN, USA).

Cytotoxic assay

The cytolytic activity of NK cells against transfected C1R cells expressing MICB*014, mutated MICB*014-Lys80, mutated MICB*014-Asp136 and untransfected target cell lines was assessed in the 4-h ^{51}Cr release assay [28]. Target cells were labelled with sodium chromate ($Na^{51}Cr_2O_4$; Invitrogen) and incubated for 4 h with NK cells in triplicate at various effector-to-target ratios. In experiments with antibody blocking, NK cells were preincubated with different amounts of the anti-NKG2D mAbs (0·3, 1, 3, 10 μg) for 1 h before addition to the target cells. Specific lysis was determined using the formula: % lysis = 100 × [(mean experimental cpm – mean spontaneous cpm)/(mean maximum cpm – mean spontaneous cpm)]. The spontaneous release of ^{51}Cr was < 25% (on average ~10%) of the maximal release. The maximum release value was determined from target cells treated with 5% (v/v) Triton X-100 (Sigma-Aldrich).

Statistical analysis

Phenotype frequencies of *MICB* were determined by number of alleles in samples/number of samples. Alleles and amino acids in case–control associations were evaluated on phenotype frequencies using the χ^2 or Fisher's exact tests by SPSS version 9 (SPSS, Inc., Chicago, IL, USA). A statistical correction in phenotype frequencies was applied for multiple comparison according to the number of alleles tested at each locus (correction factor = 9). Significance of the differences in the distributions of MICB alleles among low and high grades of the CXCA and control groups and HPV typing was tested by χ^2 as indicated by *P*-value.

Results

Major histocompatibility complex class I chain-related gene B genotyping in healthy NETF and CXCA patients

Typing of 15 *MICB* alleles and one group of alleles could be validated using our described PCR–SSP method with 29 primer mixes. The interpretation diagram for typing is shown in Fig. 1. Comparison of *MICB* allele distributions between 99 CXCA patients and 100 NETF is listed in Table 3. For NETF, seven *MICB* alleles and one group of alleles were found, of which the four most common *MICB* alleles were *MICB*00502* (31·0%), *MICB*002/007* (29·5%), *MICB*004* (11·5%) and *MICB*008* (8·5%) respectively.

Association of MICB and CXCA

According to the allele distribution in CXCA patients, eight *MICB* alleles and one group of alleles were identified. Among these alleles, *MICB*00501* was found only in CXCA patients with a frequency of 6·1% (*P*-value = 0·012). However, when the corrected *P*-value was calculated, no distinct allele was associated significantly positively with CXCA. Interestingly,

Table 3. The distributions of major histocompatibility complex class I chain-related gene B (MICB) alleles in north-eastern Thai females (NETF) and cervical cancer (CXCA).

	NETF (n = 100)		CXCA (n = 99)				
MICB	*n*	% PF	*n*	% PF	*P*-value	Pc	Odds ratio
00502	62	62·0	34	34·3	0·0001	0·0009	0·32
002/007	59	59·0	53	53·5	0·44		
004	23	23·0	26	26·3	0·59		
008	17	17·0	19	19·2	0·69		
014	7	7·0	13	13·1	0·15		
00503	6	6·0	6	6·1	0·99		
013	4	4·0	8	8·1	0·23		
003	1	1·0	3	3·0	0·31		
00501	0	0·0	6	6·1	0·0124	0·1119	
Blank†	21	21·0	30	30·3	0·13		

†Blank demonstrates the percentage of homozygous alleles in the populations.

Table 4. The amino acid substitutions of major histocompatibility complex B class I chain-related gene B (MICB) alleles in north-eastern Thai females (NETF) and cervical cancer (CXCA).

	NETF ($n = 100$)		CXCA ($n = 99$)			
Position	*n*	% PF	*n*	% PF	*P*-value	Odds ratio
Aspartic75	96	96·0	95	96·0	0·99	
Asparagine75	22	22·0	26	26·3	0·48	
Glutamic80	80	80·0	70	70·7	0·08	
Lysine80	89	89·0	71	71·7	0·0021	0·31
Isoleucine121	97	97·0	99	100·0	0·08	
Methionine121	17	17·0	19	19·2	0·69	
Aspartic136	89	89·0	71	71·7	0·0021	0·31
Asparagine136	80	80·0	70	70·7	0·13	
Isoleucine212	1	1·0	2	2·0	0·55	
Glycine291	99	99·0	94	94·9	0·09	
Serine291	10	10·0	20	20·2	0·05	

*MICB*00502* was associated negatively with CXCA, suggesting a protective allele with a corrected *P*-value of 0·0009 and an odds ratio of 0·32, as shown in Table 3. These data were re-analysed and it was found that statistically significant differences of the *MICB* allele distributions among low and high grades of CXCA and NETF were observed in *MICB*00502*. There were statistically significant differences in the distributions of this allele between NETF and low-grade (*P*-value < 0·001), and between NETF and high-grade cancer (*P*-value = 0·013). However, the differences of percentage PF between low and high grades of CXCA were not significant (*P*-value = 0·064). The data reconfirmed that *MICB*00502* was associated negatively with the disease, but not stages, of CXCA. In addition, the association between the MICB alleles and HPV typing status of patients, i.e. positive or negative HPV PCR, was not significant (*P*-value = 0·960) (data not shown).

Association of amino acid substitutions of MICB and CXCA

According to the reported alleles, there were 12 amino acid substitutions in the α1, α2 and α3 domains but only six substitutions were identified in the north-eastern Thai population containing D75N, E80K, I121M, N136D, T212I and G291S (Table 4). The *MICB*00501*, *00502* and *00503* alleles carry synonymous amino acid substitutions. Lys80 and Asp136 were associated negatively with CXCA (*P*-value = 0·0021, odds ratio = 0·3134; Table 4). It should be noted that MICB*00502 carries Lys80 and Asp136. Thus, this result is in agreement with the allele association study, suggesting that these amino acid substitutions may bind strongly to NKG2D, leading to stronger immune activation.

Amino acid substitutions of MICB affecting NKG2D induced NK cell activation

To investigate which of these positions affected NKG2D binding and activation, plasmid DNAs encoding the *MICB*014* gene was constructed by the pcDNA 3·1 Directional TOPO Expression kit. Site-directional mutagenesis was then performed to mutate amino acid position 80 from glutamic acid to lysine, called MICB*014-Lys80, and position 136 from asparagine to aspartic acid, called MICB*014-Asp136 (Table 2). These plasmid DNAs were transfected into the C1R cells to produce stable transfectants expressing the three MICB proteins. By staining with anti-MICA/B mAb (1D10), all transfectants expressed MICB on the cell surface but at different levels (Fig. 2a). In addition, the NKG2D-Ig fusion protein bindings varied substantially, with MICB*014-Asp136 transfectants representing strong binding, whereas MICB*014 and MICB*014-Lys80 were weaker (Fig. 2b). When transfected cells were cultured with NK cells and measured for the cytolytic activity using the chromium release assay, both MICB*014 and mutants could comparably induce NK cells to kill target cells (Fig. 3a). To determine whether the different binding capacity of MICB and mutated MICB to NKG2D would affect NK cell activity the MICB transfectants were co-cultured with NK cells pretreated by anti-NKG2D at different concentrations, from 0·3 to 10 μg of anti-NKG2D, to block the killing activity caused by NKG2D. Transfected cells carrying MICB*014-Asp136 was blocked by anti-NKG2D less efficiently than those carrying MICB*014-Lys80 and MICB*014 (Fig. 3b). It should be noted that MICB*014-Lys80 and MICB*014 were expressed at a comparable level on transfected cells (Fig. 2), with MICB*014-Lys80 transfectants showing a slightly weaker staining pattern. However, MICB*014 transfectants could not induce killing activity when the NK cells were pretreated with anti-NKG2D (Fig. 3). In contrast, MICB*014-Asp136 induced elevated killing activities at all concentrations of anti-NKG2D tested. This result suggested that although MICB*014 wild-type and mutants could induce NK cell killing comparably, aspartic acid at position 136, affecting NK cell-killing activity, could be revealed only by anti-NKG2D blocking experiments. This motif was carried by MICB*00502, which was associated negatively with CXCA.

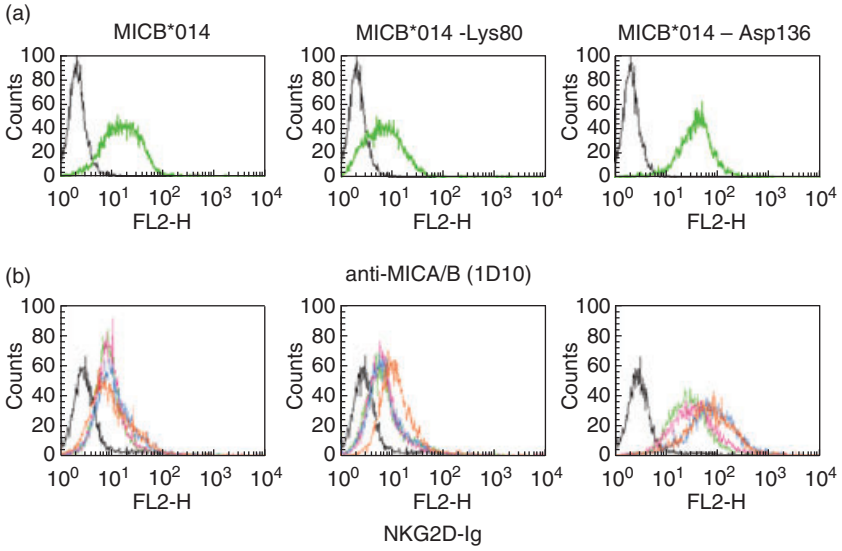

Fig. 2. Differential expression and binding of NKG2D by allelic variants of major histocompatibility complex class I chain-related gene B (MICB). Transfected C1R cells were stained with anti-MICA/B (a) and different concentrations of NKG2D-immunoglobulin (Ig) fusion protein (green, pink, blue and orange lines represent 20, 30, 40 and 50 μg/ml of NKG2D-Ig respectively) (see pdf online for colour) (b) to detect the MICB expression on the cell surface and NKG2D binding capacity. MICB*014-Asp136 could be expressed on the cell surface higher than MICB*014 or MICB*014-Lys80, and thus could bind to NKG2D-Ig more than other clones respectively.

Discussions

In this study, we have described the distribution of *MICB* alleles in NETF using PCR–SSP modified from Ahmad *et al.* [22], Gonzalez *et al.* [23] and Collins *et al.* [24] for typing of 15 *MICB* alleles and one group of alleles. However, this set could not discriminate *MICB*002* and *MICB*007* and could not identify *MICB*010*, which carries polymorphism in exon 6. Because PCR–SSP is recognized generally to be a highly robust and reproducible technique, the PCR–SSP *MICB* typing system described here will allow amplification of all *MICB* alleles and their possible combination as heterozygous alleles under identical conditions. Control DNA samples were obtained from homozygous typed cell lines (HTCLs) for method validation. The specificity of all *MICB* amplifications was verified using a set of oligoprimers combined in 29 mixtures of PCR reactions. During the development of a strategy for *MICB* typing by PCR–SSP, the similar result of inability to amplify *MICB*001* and *MICB*00504* using reference HTCLs (HELA and RML cell lines) confirmed previous observations by Gonzalez *et al.* [23] and Schroeder *et al.* [29] that the sequences of these alleles were artefacts. Thus, only 14 from 15 *MICB* alleles and one group of alleles were typed successfully. It is essential that every primer mix should be validated with standard DNA to ensure the absence of multiple amplifications and specificity. This is due to the multi-copy nature of the *MIC* gene family [12,13]. To avoid this situation, in some cases a first PCR was performed to amplify specifically a MICB fragment carrying exons 2–4 (Wongsena *et al.* in preparation). Then, the *MICB* typing was performed using this PCR product as a template.

The data of *MICB* distribution in our population are in agreement with a previous report on a Spanish population [23], that alleles *MICB*00502* (31·0%), *MICB*002/007* (29·5%), *MICB*004* (11·5%) and *MICB*008* (8·5%) are the

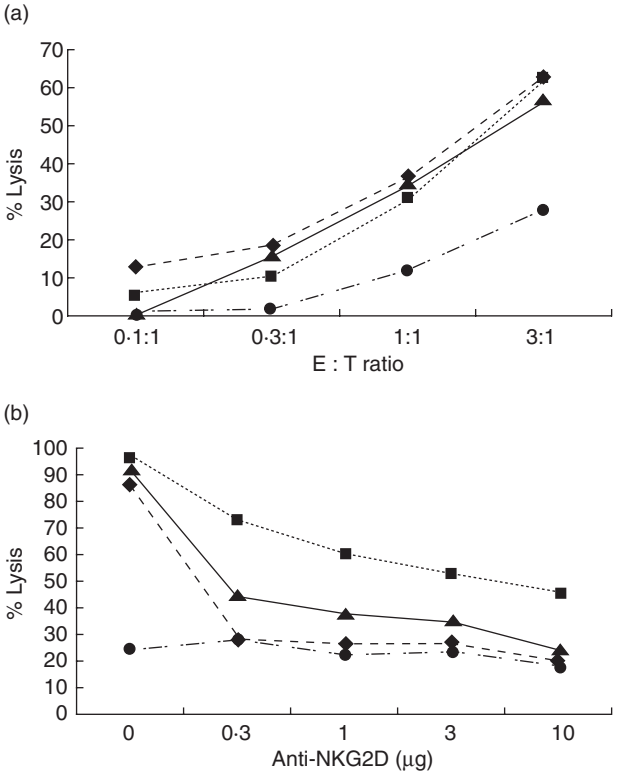

Fig. 3. Anti-NKG2D could inhibit MICB*014-Asp136 less efficiently in NKG2D-mediated lysis. (a) Major histocompatibility complex class I chain-related gene B (MICB) transfected cells were cultured with natural killer (NK) cells. Both MICB*014 wild-type and mutants could induce NK cells to kill target cells. (b) MICB transfected C1R cells were cultured with NK cells treated with different concentrations of anti-NKG2D at an effector : target ratio of 3 : 1. Although MICB*014 (▲), MICB*014-Lys80 (◆) and MICB*014-Asp136 (■) could induce NK cells to kill target cells compared with untransfected cells (●), MICB*014-Asp136 needed a high concentration of anti-NKG2D to block the activity of NK cells.

most frequent alleles. Four alleles, *MICB*006*, *MICB*009N*, *MICB*011* and *MICB*012*, have not been detected in both the CXCA group and our control population group. Even though *MICB*009N* can be found in Japanese and east Asians [30], the absence of this and other alleles suggests that these alleles are also rare in our population, as reported previously in Spain [23]. Interestingly, *MICB*00502* represented the most common allele found in the control population and was associated negatively with CXCA ($Pc = 0{\cdot}0009$), suggesting a protective *MICB* allele.

Major histocompatibility complex class I chain-related gene B is represented polymorphically by 18 alleles that variably include 12 bi-allelic amino acid substitutions with fairly random distribution in the extracellular domains [15–21]. However, there has been no evidence for functional significance of this allelic variation. Our study showed that two amino acid substitutions of MICB found in MICB*00502 (E80K and N136D) were associated negatively with CXCA. MICB*00501 and MICB*00503 also shared these substitutions, but they were not found to be associated with CXCA. This may be because they were rare in our population. Although some MICB motifs have been predicted to be involved in NKG2D binding [31], these two non-synonymous amino acid substitutions were not included. It is conceivable that these two amino acid changes could affect protein structure and/or function, as they are non-conservative. We have tested these two substitutions functionally regarding NK cell activation. Apparently, MICB*014 with aspartic acid at 136 was expressed at a higher level when compared with MICB*014 wild-type or MICB*014-Lys80. This could be the effect of stable transfectant selection or the gene sequence affecting expression level [32]. The NKG2D binding capability of these MICB proteins was in accordance with protein expression detected by antibody. Interestingly, these MICB proteins could, comparably, induce cytolytic activities of NK cells but differently in the anti-NKG2D blocking experiments. NK cells were treated with anti-NKG2D and washed before incubation with target cells. Thus, the NKG2D receptors on NK cells should be blocked at the same level regardless of the different expressions of MICB on transfectants. Consequently, the different cytolytic activities should be resulted from the kinetic differences reflected by binding affinity between NKG2D and MICB or MICB mutants, suggesting that aspartic acid at position 136 could probably induce NK cell-killing more efficiently. It is also possible that the level of MICB expression may contribute to this phenomenon and that the polymorphic residue at 136 has no effect on NK cell activation. A more sensitive method, such as the use of the Biacore system, may be needed to evaluate these differences. However, our study is the first report on the functional polymorphism of MIC proteins on NK cell activation by not only NKG2D binding [33].

In conclusion, our typing method of *MICB* alleles enabled us to show the overview picture of the *MICB* distribution in our population. The associations of MICA and haplotype analysis of *MICA* and *MICB* are presented elsewhere (Jumniansong *et al*. unpublished data). No distinct *MICB* allleles with strong positive associations indicating a risk factor for CXCA were identified, but *MICB*00502* had a significantly negative association, indicating a protective allele. This allele carries an aspartic acid at position 136 that may affect expression and NKG2D binding, leading to a higher degree of NK cell-killing activities.

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