RESEARCH ARTICLE

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Association of *MICA* Gene Polymorphism in *Opisthorchis viverrini*-Induced Periductal Fibrosis in Northeastern Thais

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Abstract

Objective: Chronic *Opisthorchis viverrini* (OV) infection is the cause of advanced periductal fibrosis (APF), subsequently leading to cholangiocarcinoma (CCA). Natural killer (NK) cells can kill hepatic stellate cells (HSCs), the initiating cells for fibrosis formation, by using the interaction between the natural killer group 2 member D (NKG2D) receptor and its ligand on the HSCs. This can inhibit the fibrosis formation. Major histocompatibility complex class I chain-related A (*MICA*) is the ligand of the NKG2D receptor and has highly polymorphic characteristics that are involved in NKG2D binding and NK cell activation. This study aimed to investigate the polymorphism of *MICA* in OV-induced fibrosis. **Method:** *MICA* typing was performed by polymerase chain reaction- sequence specific primer (PCR-SSP) and sequencing in two groups: OV infection without fibrosis (N = 99) and with fibrosis (N = 290). **Result:** Six alleles were identified and the *MICA**010 allele had the highest frequency in both groups. The *MICA**00201-02 allele was a protective factor for fibrosis (OR= 0.508, 95%CI= 0.34-0.76, Pc <0.05), while the *MICA**019 allele was suggested to be a risk allele for fibrosis (OR=1.95, 95%CI=1.25-3.03, Pc<0.005). In addition, two motifs, glycine (G) at position 14 and glutamine (Q) at position 251, were negatively associated with fibrosis (G14: OR=0.508, 95%CI=0.34-0.76, Pc <0.05 and Q251: OR=0.586, 95%CI=0.41-0.84, Pc <0.05). Moreover, the distribution of the *MICA*-129 genotype also showed the protective genotype (Pc<0.05, OR=0.319, 95%CI= 0.12-0.54) for fibrosis. The *MICA**00201-02 allele encoded all these motifs, and this suggested that it might lead to strong NK cell activation to kill HSCs, subsequently preventing fibrosis formation. **Conclusion:** This study described initial evidence suggesting that the polymorphism of the *MICA* gene might be a marker for OV-derived periductal fibrosis.

Keywords: Liver fibrosis- cholangiocarcinoma- *MICA*-129 methionine- NKG2D receptor- NK cell

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Introduction

Opisthorchis viverrini (OV) infection causes opisthorchiasis, which is a major public health problem in various regions of Asian countries, including Thailand (Sithithaworn et al., 2003, Sripa et al., 2011). Most OV infections are asymptomatic, but mild symptoms such as dyspepsia, abdominal pain, constipation, and diarrhea can occur. Chronic infection can result in chronic inflammation of the intrahepatic bile ducts, leading to periductal fibrosis due to repetitive tissue damage (Sripa et al., 2012). The fibrosis can manifest as echogenicity in the periportal area, which can be detected by ultrasonography. More than 20% of chronic infections can progress to advanced periductal fibrosis (APF) (Mairiang et al., 2017). In this case, treatment is very important because APF can serve as a risk factor for the formation of cholangiocarcinoma (CCA) (Hitanant et al., 1987, Sripa et al., 2003, Kaewpitoon et al., 2008, Sripa et., 2012, Mairiang et al., 2021).

Liver fibrosis is a complex process involving many cells. Chronic liver damage initiates the formation of fibrosis. Hepatic stellate cells (HSCs), which are located in the space between hepatocytes and sinusoidal endothelial cells, are the primary source driving the fibrogenic process (Josan et al., 2015). Additionally, natural killer cells (NK cells) are immune cells that participate in the

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response to liver fibrosis development by engaging their activating receptors, especially the NKG2D receptor (Tosello-Trampont et al., 2017). Previous studies have shown that NK cells have anti-fibrogenic properties by killing HSCs that express NKG2D ligands, such as RAE1 (in mice) (Radeava et al., 2006) and *MICA* (in humans) (Jin et al., 2017), and NK cell-derived IFN-γ can induce HSC apoptosis (Bansal et al., 2014). Thus, the engagement of NKG2D receptor and its ligand plays an important role in liver fibrosis development.

Major Histocompatibility complex class I chain-related A (*MICA*) is one of the ligands of the NKG2D receptor, which heat, viral infection, inflammation, and DNA destruction can induce *MICA* expression (Choy et al., 2010). The *MICA* gene is found in the classical class I region and is composed of 6 exons. Exons 2-4 encode the extracellular (α1- α3) domain, which is involved in NKG2D binding. The *MICA* gene is highly polymorphic, with around 100 alleles recognized by the IMGT/HLA database to date. The polymorphic positions, especially in exons 2-4, mainly involve single amino acid substitutions that affect NKG2D binding affinity and NK cell activation (Li et al., 2000, Steinle et al., 2001). Therefore, this study aims to identify the association of *MICA* gene polymorphism and amino acid substitutions that affect NKG2D binding, subsequently leading to APF in opisthorchiasis patients.

Materials and Methods

Patient samples

The DNA samples used in this study were the leftover specimen that were approved by the Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (HE641133) under the umbellar of ethic number HE480528. In brief, subjects were recruited from seven villages in Khon Kaen, Thailand, where OV transmission was high along the Chi River Basin. Opisthorchiasis patients were identified by microscopic fecal examination for OV infection which detailed description in previous report (Mairiang et a 2012, Saichua et al 2012). Then, the abdominal ultrasound examination previously described by Mairiang et al. was used to classify APF (Mairiang et al. 2012, 2021). Patients with grade 0 and grade 1 fibrosis (no echoes and echoes in one segment of liver) were classified into the APF negative group, and those with grade 2 and grade 3 fibrosis (echoes in 2, 3 and more segments of liver) were classified into the APF positive group. The patients were then divided into two groups: OV positive (OV+) and APF negative, and OV+ and APF positive. DNA was extracted from white blood cells from 99 OV+ and APF (-) samples and 290 OV+ and APF (+) samples. Finally, all patients were referred for treatment at local state hospitals.

PCR-SSP tying and sequencing for MICA polymorphisms

To identify *MICA* polymorphisms, a modified PCR-SSP method from a previous publication (Jumnainsong et al., 2007) was performed. In brief, 12 primer mixtures were used to identify *MICA* alleles with polymorphism positions in exon 2-4. These primer mixtures were specific to six *MICA* alleles, which are the most commonly occurring alleles in the Northeast Thai population and cover 86.8% of the population (Romphruk et al., 2001). The primer sequences for the 12 primer mixtures and primer checkerboard were shown in Table 1 and 2, respectively. Internal control primers, 439 base pairs (bp) human growth hormone (HGH), were also used to check PCR reactions. The HGH forward primer (5'-CAgTgCCTTCCCAACCATTCCCTTA-3') and HGH reverse primer (5'-ATCCACTCACGgATTTCTg TTgTgTTTg-3') were used. However, this primer set had some limitations as it cannot identify polymorphisms in the transmembrane region and some rare groups of alleles.

One hundred ng/μL of genomic DNA template was required for PCR. The PCR mixture was consisted of 1 mM dNTP, 25 mM MgCl2, 10X PCR buffer, 5 U Taq DNA polymerase enzyme (all from Vivantis, USA), 0.25 μM of each specific primer, and 0.1 μM of control primers. For PCR, the Veriti 96-well Thermal cycler (Applied Biosystem, USA) was used. The following series of thermal cycling conditions were programmed: initial denaturation at 96°C for 2 minutes; 5 cycles of denaturation at 96°Celsius (°C) for 30 second (s), annealing at 68°C for 60 s, and extension at 72°C for 40 s; 21 cycles of denaturation at 96°C for 30 s, annealing at 65°C for 60 s, and extention at 72°C for 40 s; 4 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 75 s, and extension at 72°C for 120 s, and final extension at 72°C for 10 minutes. Gel electrophoresis was used to confirm the 13 μl amplification product. A 1.5% agarose gel (Vivantis, Malaysia) was stained with ethidium bromide and visualized using an ultraviolet (UV) transilluminator (Shimadzu, Japan).

If the samples could not be fully identified by the 12-primer mixture set, the sequencing method was applied to solve the issue. *MICA* exon 2-3 was amplified with the primers *MICA* 2-3F (5' TGAAATCCTCTTCTTGTCCCTTTGC 3') and *MICA* 2-3R (5' AGGGTCCTCTACTTGCCCTGATTAC 3') before sequencing (Toledo-Stuardo et al., 2021). There were 21 samples which showed only one allele by PCR-SSP. These samples were amplified for the sequencing, and the result of *MICA* typing by PCR-SSP and sequencing was the same (table S1).

Amino acid substitution selection

The amino acid sequence in α1- α3 domain of common 6 *MICA* alleles in the population was aligned to identify the amino acid position that difference among 6 alleles. This demonstrated 13 non-synonymous amino acid substitutions in these domains (table S2). Since α1- α3 domain of *MICA* are the extracellular domain that bind to NKG2D receptor, these amino acid substitutions can affect NKG2D binding and NK cell activation.

Statistical analysis

The *MICA* allele frequencies were calculated by direct counting. Statistical analysis was performed using IBM Statistics SPSS software (version 26). To determine the significance of the association between groups, they were compared using the Pearson chi-square method, and p-values and corrected p-values were calculated.

To determine the disease risk in carriers of specific alleles, odds ratios (OR) and 95% confidence intervals (95%CI) were calculated using Woolf's method. Bonferroni's multiple correction (corrected p-value, Pc) was obtained by multiplying the p-value with the number of tests performed, and a Pc value <0.05 was considered statistically significant.

Results

Clinicopathological features of subjects

According to the abdominal ultrasound examination in opisthorchiasis patients, they were classified into fibrosis and non-fibrosis groups. In the fibrosis group, males were more prevalent than females (62.8% and 37.2%, respectively). The occurrence of non-fibrosis by sex was nearly the same. The average age was 45.5 years in the non-fibrosis group and 48.1 years in the fibrosis group. In addition, other factors involving liver fibrosis; smoking and alcohol drinking were not significant difference (Table 3).

Association of MICA phenotypes in non-fibrosis and fibrosis groups of opisthorchiasis

In both groups, six alleles were identified, and *MICA**010 was the most common allele. The association between *MICA* phenotypes in the non-fibrosis and fibrosis groups was calculated using the Pearson chi-square test. These p-values were further corrected by multiplying them by the number of alleles detected and tested in this population. According to Table 4, the distribution frequency of the *MICA**00201-02 allele was significantly lower in fibrosis patients than in non-fibrosis patients. This suggested that *MICA**00201-02 was a protective factor against fibrosis (24.8% vs. 14.3%, OR=0.51, 95%CI=0.34-0.76, Pc < 0.05). Another allele, the *MICA**019 allele, was suggested as a risk allele because it was significantly higher in OV-induced fibrosis than in non-fibrosis OV patients (14.1% vs. 24.3%, OR=1.95, 95%CI=1.25-3.03, Pc<0.005).

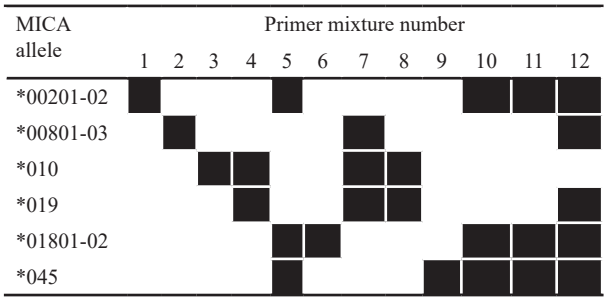
Association of MICA genotypes in non-fibrosis and fibrosis groups of opisthorchiasis

As shown in Table 5, the distribution frequency of *MICA**00201-02 homozygotes were significantly higher in the non-fibrosis group than in the fibrosis group, which may be a protective genotype against fibrosis. Similarly, *MICA**010 homozygous alleles also had a negative association.

Table 1. Twelve Primer Mixes and Primer Sequences for *MICA* Genotyping

Mix No.	Name and primer sequences (5'- 3')	Exon	Positon	Target allele
1	2; ACCTCACggTgCTgTCCg	2	22-40	00201-02, 011, 015,017, 030, 034, 035, 041
	14.2: CCCAgCATTTCTACTACgATA	3	319-340	
	2': CTCAggACTACgCCggATTT	3	517-537	
2	8: ACggCgATATCTAgAATCCg	3	503-523	00801-03, 035, 037, 039, 042
	8': TCCTgACgCCTggTCAgTA	4	638-657	
3	10: CAgAgCCCCACAgTCTTCC	2	1-17	10
	13': TCTggAggACTggggCAAC	3	385-403	
4	20: TACggCgATATCTAgAATCCA	3	502-523	010, 016, 019, 022, 033
	8': TCCTgACgCCTggTCAgTA	4	638-657	
5	4.2: TCAgCCCTTCCTgCgCTA 2': CTCAggACTACgCCggATTT	2 3	89-107 517-537	001, 00201-02, 00701-02, 011, 01201-02, 015, 017, 01801-02, 021, 029, 030, 034, 035, 037-039, 041, 043, 045
6	16: CATgggACAgAgAgACCAgA	2	174-192	001, 01201-02, 01801-02, 021
	2': CTCAggACTACgCCggATTT	3	517-537	
7	4: gTCAgCCCTTCCTgCgCTA	2	90-107	004-006, 00801-03, 00901-02, 010, 016, 019, 024, 025, 028, 031, 033, 042, 044
	12': CTgCATGCATAgCgTgATAgT	3	467-488	
8	4: gTCAgCCCTTCCTgCgCTA	2	90-107	004, 006, 00901-02, 010, 016, 019, 031, 033, 044
	25': gTTCTCCTCAggACTACgCT	3	523-543	
9	573FT: ATggTgAATggT5CACCCgCAgT	4	552-573	45
	752RC: gAACCTCTgCTCCTCTCCTTC	4	752-773	
10	4.2: TCAgCCCTTCCTgCgCTA 373R: TggggCAttgTCCATTCCTC	2 3	89-107 373-393	00201, 00701-02, 011, 01201-02, 013-015, 017, 01801-02, 021, 022, 029, 030, 034-039, 041, 043, 045
11	272F: CAggCTTgCATTCCCTCCA	3	253-272	001, 00201-02, 005, 00701-02, 011, 01201-02, 013-015, 01801-02, 021, 022, 029, 030, 034-039, 041, 043, 045
	19': AgCCCTgCATgTCACggTA	4	594-613	
12	86FG: CAgAgCCCCACAgTCTTCg	2	1-17	All except 010
	13':TCTggAggACTggggCAAC	3	385-403	

Table 2. A Primer set of *MICA* Typing Consisting of 12 Primer Mixes



Shaded boxes are positive reactions; All of these alleles were the common alleles in Northeast Thai population and covered around 86.8% of the population.

Association of MICA amino acids substitutions in non-fibrosis and fibrosis groups of opisthorchiasis patients

Since amino acid substitutions are involved in NKG2D binding affinity and lead to the activation of immune cells, we analysed 13 positions of non-synonymous amino acid substitutions in exons 2-4 of 6 *MICA* alleles in this population (Table S2). Two positions, Glycine-14 and glutamine-251, were negatively associated with fibrosis (Pc = 0.004 and 0.044, respectively, Table 6). Additionally, a previous study showed that amino acid position 129, which is either methionine or valine, was an important position because it affected NKG2D affinity [14]. Although the association of the allele frequency of position 129 was not significant (Table 6), the distribution of *MICA*-129 genotypes indicated that the *MICA*-129 Met/Met homozygous genotype had a negative association and may be assumed as a protective genotype (Pc < 0.05, OR = 0.319, 95%CI = 0.16-0.65) for fibrosis (Table 7).

Discussion

Chronic OV infection can lead to advanced periductal fibrosis and, ultimately, cholangiocarcinoma (Sripa et al., 2012). Numerous immune cells are involved in the fibrosis mechanism, with NK cells playing a critical role through NKG2D receptor activation, with *MICA* as an important ligand for this receptor. *MICA* gene is highly polymorphic, particularly in the extracellular domain region, resulting in amino acid substitutions that affect the affinity of NKG2D binding (Li et al., 2000, Steinle et al., 2001). This is the first report on the association study of extracellular domain polymorphism and periductal fibrosis derived from OV infection. PCR-SSP was the technique in this study and it has some limitations. After PCR-SSP with primer mix number 1 to 11, there were 54 samples that positive with primer mix number 3, 4, 7 and 8. The interpretation of these samples was *MICA**010 but cannot exclude *MICA**019. To solve this problem, primer mix number 12 was established to identify homozygous *MICA**010 and heterozygous *MICA**010 and *MICA**019. This primer could be positive with all allele except *MICA**010. There were 13 samples shown negative with this primer. The interpretation of 13 samples was the homozygous *MICA**010. In contrast, 41 samples were positive with this primer. They were the heterozygous *MICA**010 and 019. Moreover, another limitation of PCR-SSP was unexpected allele. After PCR-SSP, only one *MICA* allele was identified in 21 samples. Thus, these samples were confirmed by the sequencing. The sequencing result demonstrated the homozygous allele that was the same result with PCR-SSP. Thus, PCR-SSP was suitable for the screening technique

Table 3. Clinicopathological Features of the Subjects

Characteristics	No-fibrosis Patients	Fibrosis Patients	Total	P-value
Number(N)	99	290	389	
Gender (%)				ns
Male	49 (49.5)	182 (62.8)	231	
Female	50 (50.5)	108 (37.2)	158	
Age (years)				ns
Range	20-59	21-60		
Average	45.5	48.1		
Intervals				
≤30	3 (3.0)	26 (9.0)	29	
31-40	10 (10.0)	64 (22.1)	74	
41-50	47 (47.5)	92 (31.7)	139	
≥51	39 (39.5)	108 (37.2)	147	
Smoking (%)				ns
Non-smoking	57 (57.6)	102 (35.2)	169	
Smoking	31 (31.3)	147 (50.7)	178	
No information	11 (11.1)	41 (14.1)	52	
Alcohol drinking (%)				ns
Never/ Occasional	72 (71.7)	155 (53.5)	227	
Regular	16 (16.2)	94 (32.4)	110	
No information	11 (11.1)	41 (14.1)	52	

ns, not significant

Table 4. *MICA* Allele Frequencies in Non Fibrosis (99) and Fibrosis (290) of Opisthorchiasis Groups

MICA alleles	OV (+), Fib (-)		OV (+), Fib (+)						
	Number 2n=198	% AF	Number 2n=580	% AF	Chi square	P -value	Pc	Odds ratio	95%CI
*00201-02	49	24.8	83	14.3	11.414	0.0007	0.004	0.508	0.34-0.76
*00801-03	36	18.2	109	18.8	1.899	0.168	NS	1.041	0.68-1.58
*010	55	27.8	153	26.4	0.147	0.701	NS	0.932	0.65-1.34
*01801-02	17	8.59	48	8.28	0.019	0.892	NS	0.961	0.54-1.71
*019	28	14.1	141	24.3	8.977	0.0027	0.016	1.95	1.25-3.03
*045	13	6.57	46	7.93	0.393	0.531	NS	1.226	0.65-2.32

NS, not significant; Significant difference, Pc<0.05

Table 5. *MICA* Genotypes Frequencies in Non-Fibrosis (99) and Fibrosis (290) of Opisthorchiasis Groups

MICA alleles	OV (+), Fib (-)		OV (+), Fib (+)		Chi square	P value	Pc value	Odds ratio	95%CI
	Number N=99	% AF	Number n=290	% AF					
*00201-02/*00201-02	5	5.05	1	0.34	10.762	**0.001**	**0.021**	**0.065**	**0.008-0.56**
*00201-02/*00801-03	7	7.07	13	4.48	1.014	0.314	NS	0.617	0.24-1.59
*00201-02/*010	15	15.15	33	11.38	0.971	0.324	NS	0.719	0.37-1.39
*00201-02/*019	9	9.09	25	8.62	0.02	0.886	NS	0.943	0.42-2.10
*00201-02/*01801-02	5	5.05	4	1.38	4.324	0.038	NS	0.266	0.07-1.00
*00201-02/*045	3	3.03	6	2.07	0.302	0.583	NS	0.676	0.17-2.76
*00801-03/*00801-03	1	1.01	1	0.34	0.639	0.424	NS	0.339	0.02-5.47
*00801-03/*010	10	10.1	44	14.83	2.097	0.148	NS	1.741	0.82-3.71
*00801-03/*019	5	5.05	37	12.41	7.204	0.0073	NS	4.535	1.36-15.07
*00801-03/*01801-02	4	4.04	5	1.72	0.625	0.429	NS	0.561	0.13-2.39
*00801-03/*045	3	2.02	6	2.07	0.001	0.976	NS	1.025	0.20-5.16
*010/*010	9	9.09	4	1.38	13.588	**0.0002**	**0.004**	**0.14**	**0.04-0.47**
*010/*019	4	4.04	37	12.76	1.579	0.209	NS	1.664	0.75-3.71
*010/*01801-02	4	4.04	16	5.52	0.983	0.322	NS	1.869	0.53-6.55
*010/*045	3	3.03	16	5.52	2.045	0.153	NS	2.832	0.64-12.54
*019/*019	2	2.02	7	2.76	0.161	0.689	NS	1.376	0.29-6.59
*019/*01801-02	3	3.03	16	5.17	1.755	0.185	NS	2.645	0.59-11.78
*019/*045	3	3.03	12	4.14	0.954	0.329	NS	2.094	0.46-9.52
*01801-02/*01801-02	1	1.01	1	0.34	0.639	0.424	NS	0.339	0.02-5.47
*01801-02/*045	2	2.02	5	1.72	0.2	0.655	NS	0.678	0.12-3.76
*045/*045	1	1.01	1	0.34	0.639	0.424	NS	0.339	0.02-5.47

NS, not significant; Significant difference, Pc<0.05

Table 6. The Allele Frequency of MICA Aamino-Acids Substitutions in Non-Fibrosis (99) and Fibrosis (290) of Opisthorchiasis Groups

Positions	OV (+), Fib (-)		OV (+), Fib (+)		Chi square	P- value	Pc	Odds ratio	95%CI
	Number 2n=198	% AF	Number 2n=580	% AF					
Proline 6	55	27.78	153	26.38	0.147	0.701	NS	0.932	0.65-1.34
Arginine 6	143	72.22	427	73.62	0.147	0.701	NS	1.073	0.75-1.54
Glycine 14	49	24.75	83	14.31	11.414	0.0007	0.004	0.508	0.34-0.76
Tryptophan 14	149	75.25	497	85.69	11.414	0.0007	0.004	1.969	1.32-2.93
Threonine 24	17	8.59	48	8.28	0.019	0.892	NS	0.961	0.54-1.71
Alanine 24	181	91.41	532	91.72	0.019	0.892	NS	1.041	0.58-1.86
Cysteine 36	79	39.9	177	30.52	5.885	0.015	NS	0.662	0.47-0.92
Tyrosine 36	119	60.1	403	69.48	5.885	0.015	NS	1.512	1.08-2.11
Methionine 129	79	39.9	177	30.52	5.885	0.015	NS	0.662	0.47-0.92
Valine 129	119	60.1	403	69.48	5.885	0.015	NS	1.512	1.08-2.11
Lysine 173	79	39.9	177	30.52	5.885	0.015	NS	0.662	0.47-0.92
Glutamic acid 173	119	60.1	403	69.48	5.885	0.015	NS	1.512	1.08-2.11
Glycine 175	115	58.08	286	49.31	4.546	0.033	NS	0.702	0.51-0.97
Serine 175	83	41.92	294	50.69	4.546	0.033	NS	1.424	1.02-1.97
Glycine 206	79	39.9	177	30.52	5.885	0.015	NS	0.662	0.47-0.92
Serine 206	119	60.1	403	69.48	5.885	0.015	NS	1.512	1.08-2.11
Tryptophan 210	79	39.9	177	30.52	5.885	0.015	NS	0.662	0.47-0.92
Arginine 210	119	60.1	403	69.48	5.885	0.015	NS	1.512	1.08-2.11
Threonine 213	79	39.9	177	30.52	5.885	0.015	NS	0.662	0.47-0.92
Isoleucine 213	119	60.1	403	69.48	5.885	0.015	NS	1.512	1.08-2.11
Serine 215	79	39.9	177	30.52	5.885	0.015	NS	0.662	0.47-0.92
Threonine 215	119	60.1	403	69.48	5.885	0.015	NS	1.512	1.08-2.11
Arginine 251	119	60.1	403	69.48	8.578	0.0034	0.044	1.706	1.19-2.44
Glutamine 251	66	33.33	131	22.59	9.016	0.0034	0.044	0.586	0.41-0.84
Glutamic acid 251	13	6.57	46	7.93	2.799	0.094	NS	1.783	0.90-3.53

NS, not significant; Significant difference, Pc<0.05

Table 7. *MICA* Position 129 Genotype Frequency in Non-Fibrosis (99) and Fibrosis (290) of Opisthorchiasis Groups

Positions	OV (+), Fib (-)		OV (+), Fib (+)		Chi square	P -value	Pc	Odds ratio	95%CI
	Number n=99	% F	Number n=290	% F					
MICA-129 Met/Met	17	17.2	18	6.2	10.837	0.0009	0.0027	0.319	0.16-0.65
MICA-129 Met/Val	45	45.5	141	48.6	0.297	0.586	NS	1.136	0.72-1.79
MICA-129 Val/Val	37	37.4	131	45.2	1.829	0.176	NS	1.381	0.86-2.20

NS, not significant; Significant difference, Pc<0.05

to identify *MICA* alleles.

There were many factors that may affect to fibrosis, such as smoking and alcohol assumption, these might be the confounding factors in this study. However, when these factors were analyzed, these factors were not significant difference between non fibrosis and fibrosis patient. In addition, there were other factors involving the development of fibrosis that were not analyzed. This was the limitation of this study.

The most common six *MICA* alleles in the Thai population were identified and analyzed in OV-infected patients with and without fibrosis. The analysis indicated that *MICA**00201-02 and *MICA**019 alleles showed correlation between the fibrosis group and non-fibrosis group of infected patients. The OV-infected fibrosis group showed that the *MICA**019 allele had a significantly higher frequency than that in the no-fibrosis group, making this allele the susceptibility effect for fibrosis. In contrast, the *MICA**00201-02 allele was the protective allele for fibrosis, especially in the homozygous *MICA**00201-02. These results differed from the association between *MICA* allele and Schistosoma-derived liver fibrosis (Gong et al., 2012) where *MICA* alleles were not significantly different between healthy and Schistosoma-derived liver fibrosis. Our results were also different from those of fibrosis derived from fatty liver (Karrar et al., 2020). Furthermore, our results differed from a previous study which showed that *MICA**002 was the risk allele for pulmonary fibrosis (Aquino-Galvez et al., 2009), which may be due to distinct ethnic groups and fibrosis mechanisms.

MICA gene polymorphisms lead to amino acid substitutions that are involved in the activation of NK cells via NKG2D binding. The amino acid substitutions among six *MICA* alleles were analyzed, and the analysis indicated that tryptophan (W) to glycine (G) at codon 14 (W14G) and glutamine (Q) at position 251 (Q251R) were negatively associated with fibrosis. These substitutions were found in extracellular domains 1 and 3 of *MICA**00201-02, which might enhance the affinity of NKG2D binding, leading to strong NK cell activation to kill HSCs and subsequently reduce fibrosis. Additionally, a previous study demonstrated that methionine at amino acid position 129 in extracellular domain 2 was involved in a strong affinity for NKG2D binding (Li et al., 2000). We found that the *MICA*-129 Met/Met genotype was significantly less prevalent in fibrosis patients compared to non-fibrosis patients, implying that the *MICA*-129 Met/Met genotype might give highly affinity of NKG2D binding and strong NK cell activation to destroy HSCs, thus playing an important role in reducing fibrosis. Since *MICA**00201-02 encodes methionine at amino acid position 129, this was another factor supporting the negative association of this allele. Interestingly, a previous study showed that valine at amino acid position 129 showed the opposite result when compared with methionine. It was involved in weak affinity to NKG2D binding (Li et al., 2000). *MICA**019 codes for valine at position 129, so *MICA**019 may be the weakly affinity allele for NKG2D receptor, leading to less NK cell activation to kill HSCs. This may be a factor supporting the positive association of *MICA**019 and promoting fibrosis. IFN-γ-producing NK cells have been demonstrated to inhibit fibrosis by inducing HSC apoptosis (Bansal et al., 2014). *MICA**00201-02, which carries 129-methionine, might strongly activate NK cells to produce IFN-γ and enhance HSC apoptosis, subsequently reducing liver fibrosis. The homozygous *MICA**010 demonstrated a protective effect on fibrosis which similar with *MICA**00201-02. This allele carried *MICA**129 Val that was opposite with *MICA**00201-02. This might be suggested that another *MICA* amino acid positions might also affect the NKG2D binding and NK cell activation, especially at amino acid position 6 that *MICA**010 was difference from other alleles. However, the exact interaction between NKG2D receptor and *MICA* in OV-derived liver fibrosis still needs further investigation. *MICA* allele might be a marker for fibrosis formation, which is an important marker for cholangiocarcinoma.

In conclusions, the *MICA**00201-02 allele had a protective effect, while the *MICA**019 allele had a susceptibility effect for OV-induced fibrosis. Three amino acid substitutions were found to be negatively associated with fibrosis. According to a previous publication, methionine-129 had a stronger affinity to NKG2D than valine-129. *MICA**00201-02 encoded all of the substitutions, especially the 129-methionine substitution. This might be the factor that supported the negative association of this allele, as it could highly activate NK cells to kill HSCs expressing *MICA*. Therefore, the *MICA* allele and amino acid substitutions could be markers for OV-induced fibrosis, which is the initiation step of cholangiocarcinoma.

Author Contribution Statement

Initiate the project: Amonrat Jumnainsong, Chanvit Leelayuwat; Collected the data and contributed data analysis: Tay Zar Myo Oo, Prasert Saichua, Paiboon Sithithaworn, Eimorn Mairiang, Banchob Sripa, Amonrat Jumnainsong; Setting the method: Tay Zar Myo Oo, Wisitsak Phoksawat; Wrote the manuscript: Tay Zar

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Ethical Declaration

This study was approved by the Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (HE641133) under the umbellar of ethic number HE480528.

Conflict of Interest

The authors have declared that no conflict of interests.

References

Aquino-Galvez A, Pérez-Rodríguez M, Camarena A, et al (2009). *MICA* polymorphisms and decreased expression of the *MICA* receptor NKG2D contribute to idiopathic pulmonary fibrosis susceptibility. *Hum Genet*, **125**, 639-48.

Bansal R, Prakash J, De Ruiter M, et al (2014). Interferon gamma peptidomimetic targeted to hepatic stellate cells ameliorate acute and chronic liver fibrosis in vivo. *J Control Release*, **179**, 18-24.

Choy MK, Phipps ME (2010). *MICA* polymorphism: biology and importance in immunity and disease. *Trends Mol Med*, **16**, 97–106.

Gong z, Luo QZ, Lin L, et al (2012). Association of *MICA* gene polymorphisms with liver fibrosis in schistosomiasis patients in the Dongting Lake region. *Braz J Med Biol Res*, **45**, 222–9.

Hitanant S, Tan-Ngarm Trong D, Damrongsak C, et al (1987). Peritoneoscopic findings in 203 patients with *Opisthorchis viverrini* infection. *Gastrointestinal Endoscopy*, **33**, 18–20.

Jin H, Jia Y, Yao Z, et al (2017). Hepatic stellate cell interferes with NK cell regulation of fibrogenesis via curcumin induced senescence of hepatic stellate cell. *Cell Signal*, **33**, 79–85.

Josan S, Billingsley K, Orduna J, et al (2015). Assessing inflammatory liver injury in an acute CCl4 model using dynamic 3D metabolic imaging of hyperpolarized [1-(13) C] pyruvate. *NMR Biomed*, **28**, 1671–77.

Jumnainsong A, Romphruk AV, Jearanaikoon P, et al (2007). Association of polymorphic extracellular domains of *MICA* with cervical cancer in northeastern Thai population. *Tissue Antigens*, **69**, 326-33.

Kaewpitoon N, Kaewpitoon SJ, Pengsaa P, et al (2008). *Opisthorchis viverrini*: The carcinogenic human liver fluke. *World J Gastroenterol*, **14**, 666–74.

Karrar A, Rajput B, Hariharan S, et al (2020). Major Histocompatibility Complex Class I Related Chain A Alleles and Histology of Nonalcoholic Fatty Liver Disease. *Hepatol Commun*, **30**, 63-73.

Li Z, Groh V, Strong RK, et al (2000). A single amino acid substitution causes loss of expression of a *MICA* allele. *Immunogenetics*, **51**, 246–8.

Mairieng E., Laha T, Bethony JM, et al (2012). Ultrasonography assessment of hepatobiliary abnormalities in 3359 subjects with *Opisthorchis viverrini* infection in endemic areas of Thailand. *Parasitol Int*, **61**, 208-11.

Mairiang E (2017). Ultrasonographic features of hepatobiliary pathology in opisthorchiasis and opisthorchiasis-associated cholangiocarcinoma. *Parasitol Int*, **66**, 378-82.

Mairiang E, Laha T, Kaewkes S, et al (2021). Hepatobiliary morbidities detected by ultrasonography in *Opisthorchis viverrini*-infected patients before and after praziquantel treatment: a five-year follow up study. *Acta Trop*, **217**, 105853.

Radaeva S, Sun R, Jaruga B, et al (2006). Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners *Gastroenterology*, **130**, 435-52.

Romphruk AV, Naruse TK, Romphruk A, et al (2001). Diversity of *MICA* (PERB11.1) and HLA haplotypes in Northeastern Thais. *Tissue Antigens*, **58**, 83–9.

Saichua P, Sithithaworn P, Jariwala AR, et al (2013). Microproteinuria during *Opisthorchis viverrini* infection: a biomarker for advanced renal and hepatobiliary pathologies from chronic opisthorchiasis. *PLoS Negl Trop Dis*, **23**, e2228.

Sithithaworn P, Haswell-Elkins M (2003). Epidemiology of Opisthorchis viverrine. *Acta Trop*, **88**, 187–94.

Steinle A, Li P, Morris DL, et al (2001). Interactions of human NKG2D with its ligands *MICA*, MICB, and homologs of the mouse RAE-1 protein family. *Immunogenetics*, **53**, 279–87.

Sripa B (2003). Pathobiology of opisthorchiasis: an update. *Acta Trop*, **88**, 209–20.

Sripa B, Bethony JM, Sithithaworn P, et al (2011). Opisthorchiasis and Opisthorchis- associated cholangiocarcinoma in Thailand and Laos. *Acta Trop*, **120**, 158-68.

Sripa B, Brindley PJ, Mulvenna J, et al (2012). The tumorigenic liver fluke *Opisthorchis viverrini* –multiple pathways to cancer. *Trends Parasitol*, **28**, 395–407.

Toledo-Stuardo K, Andrea Canals MM, Rodríguez-Siza VGJ, et al (2021). Major Histocompatibility Complex Class I-Related Chain A (*MICA*) Allelic Variants Associate with Susceptibility and Prognosis of Gastric Cancer. *Front Immunol,* **12**, 12-7.

Tosello-Trampont A, Surette FA, Ewald SE, et al (2017). Immunoregulatory Role of NK Cells in Tissue Inflammation and Regeneration. *Front Immunol*, **20**, 301-8.