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EVALUATE THE POTENTIAL SNPs FOR BREEDING SELECTION OF WHITE SPOT SYNDROME VIRUS RESISTANCE IN *LITOPENAEUS VANNAMEI*

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SUMMARY

White spot syndrome virus (WSSV) is a dangerous virus causing great damage to shrimp farming. Many genes related to disease resistance mechanisms have been identified and studied. In this study, we amplified and sequenced nine fragments of DNA harboring SNPs in the genes involved in WSSV resistance of white-legged shrimp, *Litopenaeous vannamei*. Allele frequencies at the SNP loci were recorded and calculated by SPSS statistical software (version 22) in the study groups: the disease shrimp (were infected with WSSV and died) and the resistance shrimp (were infected with WSSV but survived). Six SNPs (in *AIF*, *ALF1*, *HAE*, *P53*, *Rab5B*, and *TRAF6* genes) were in accordance with Hardy–Weinberg Equilibrium (HWE) ($p > 0.05$) while three SNPs (in *ALF2*, *BGB*, and *CAL*) were not ($p < 0.05$). For *AIF* and *ALF1* genes, the frequencies of GG and CC genotypes were significantly different between two groups and were associated with an decreased resistance with WSSV compared to the AA and TT genotypes ($p = 0.021$ and $p = 0.017$, respectively). The G and C alleles were associated with a decreased resistance with WSSV ($p = 0.000$ and $p = 0.001$, respectively). For *HAE* gene, the frequency of TT genotype was significantly different between two groups and was associated with a increased resistance with WSSV compared to the TC+CC genotype (OR = 68.750; 95% CI: 11.462–412.381; $p = 0.000$). For *Rab5B* gene, the frequency of CC genotype was significantly different between two groups and was associated with an increased resistance with WSSV compared to the TT genotype in all three tested models ($p<0.05$). The C allele was associated with an increased resistance with WSSV (OR = 3.974; 95% CI: 1.754–9.003; $p = 0.001$). The above evaluation results suggested that the potential SNPs of these *AIF*, *ALF1*, *HAE*, and *Rab5B* genes can be used as the molecular markers for breeding selection the resistance to white spot disease in white-legged shrimp *L. vannamei*.

Keywords: *Litopenaeus vannamei*, molecular marker, SNP, WSSV resistancy

INTRODUCTION

Aquaculture is a major economic resource in many countries. However, aquaculture in general and shrimp farming in particular has been dramatically affected by many pathogenic diseases, mainly caused by viruses. In these viruses, the virus that causes white spot disease (WSSV – White Spot Syndrome Virus) and serious damages to the shrimp farming, is a dangerous virus. To combat the viruses, shrimp has formed many mechanisms against the invasion and spread of the virus with the participation of many different genes. Disease resistance mechanisms known as enhancing the expression and activating of the genes in the immune system or the genes play a part in the signaling pathways that activate the activity of target genes to fight the infection of viruses and the genes associated in the apoptosis of cell to limit the spread of the virus…

A large number of genes, including genes coding for proteins of the Toll/IMD-NF-kB signaling pathway (Spatzle/Tolls/MyD88/Pell/TRAF6/Dosal), small G proteins, pattern recognition receptors (C-type lectin), apoptosis proteins (AIF, caspases, CYC, P53…), proteins implicated in immune response (cathepsin, HSP, lysozyme)… have been studied.

Research of Liu *et al.* (2016) showed high transcript levels of the *Toll*, *IMD*, *Pelle*, *IAP1*, *TRAF6*, *ALF* genes and suggested that the expression of these genes plays an important role in Toll/IMD signaling pathway. Function of TRAF6 (tumor necrosis factor receptor associated factor 6) has been studied in the immune responses of invertebrates and showed that TRAF6 was adjusted up to 2.7 folds in the hepatopancreas after 3 hours infected with WSSV. This finding indicated that TRAF6 associated with antimicrobial responses through the regulation of expression of *AMP* genes (Wang *et al*., 2011). Zhang *et al.* (2012) reported that MyD88 plays a role in antimicrobial and antiviral responses in *L. vannamei*. Li *et al*. (2017) identified the role of p53 in the adjustment of immediate-early (IE) of antimicrobial peptide (AMPs) when *L. vannamei* infected with WSSV. These antimicrobial peptides include anti-lipopolysaccharide factors (ALF1, ALF2), crustin, lysozyme and penaeidin. Studies have shown the enhanced expression of ALF when WSSV infection shift from latent to acute phase (Li *et al*., 2013). Zhao *et al*. (2015) found that Rab5, Rab6, Rab7 (the members of small G protein family) proteins were over-expressed in shrimp after WSSV infection. The results demonstrated the role of these proteins in the immune responses of shrimp. Besides, the vascular endothelial growth factors (VEGF) that take part in the promotion of cell growth, cell migration, vascular enhancement, vascular formation, and the reactions between host cell and pathogen contribute to confer resistance to shrimp against WSSV (Tammela *et al*., 2004).

Apoptosis is often considered as a type of antiviral immune response. This is an important defense mechanism of cell to suppress the replication and spread of virus (Everett, McFadden, 1999; Koyama *et al*., 2000). This process occurs at early stage after viral infection and before viral replication leading to the production and the spread of virus would be severely impacted. AIF, caspases, and cytochrome c (CYC) are three factors involved in the onset of apoptosis (Marsden *et al*., 2002). A special fragment on the caspase gene (fragment 3) has been identified in WSSV-resistant shrimp. The expression level of five caspase genes were reported that are highly sensitive to infection with WSSV. The expression of *AIF*, *CYC*, and caspase 3 genes were significantly increased in WSSV-infected shrimps. The results suggested that *AIF*, *CYC*, and caspase 3 genes plays an important role in immune responses against WSSV infection (Hu, Yao, 2016). In shrimp, ROS (reaction oxygen species) are produced during defense reactions and responds to virus infection (Ji *et al*., 2011). ROS are then rapidly eliminated by antioxidant enzymes, which may participate in the production of reactive oxygen compounds used in the destruction of pathogens (Campa-Córdova *et al*., 2002). When the balance between the production and elimination of ROS is disturbed, excessive production of ROS leads to cellular damage and subsequently apoptosis (Legeay *et al*., 2005). In addition, inhibitor of apoptosis (IAP) protein family members also participate in many cellular processes such as apoptosis and response to pathogens. Leu and colleagues (2012) evaluated that IAP1 plays a major role in the regulation of apoptosis of shrimp.

In this study, we evaluated the SNPs in some genes that related to WSSV resistance to identify the potential SNPs for the selection breeding of white-legged shrimp.

MATERIALS AND METHODS

Shrimp samples including healthy (uninfected with WSSV, 30 samples), disease (infected with WSSV and died, 30 samples), and resistance samples (infected with WSSV and survived, 30 samples) were collected from Research Institute for Aquaculture No. 3.

Total DNA of shrimp samples were extracted by Gene JET Genomic DNA Purification kit (Thermo, USA). The extraction steps were performed according to the manufacturer's instructions.

PCR reactions were carried out using 10 pmol of each primer (Table 1) and 20 ng of DNA as template in 25 µl reactions containing 1X Dream Taq buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1U Dream Taq (Thermo, U.S.A.). The cycle conditions were 95°C for 1 min and 35 cycles of 95°C for 10 s, 58°C – 62°C for 30 s and 72°C for 30 s, followed by a final extension step of 72°C for 10 min. The PCR amplification was carried out on an Eppendorf Mastercycler EP gradient (USA Scientific, Inc).

The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Germany) and sequenced directly using an ABI PRISM 3500 (U.S.A.). The sequences were aligned and compared to the reference sequences in GenBank using BioEdit software version 7.2.5.

Results were recorded and analyzed by SPSS statistics software (version 22) to calculate the

frequency of alleles among the study groups. Chi-squared test ($\chi 2$) was used to test whether allele distribution of each SNP follows Hardy–Weinberg Equilibrium (HWE). Three models (additive, dominant, recessive) for associations of the studied SNPs with WSSV resistance were tested. The normal distribution was used to estimate the confidence interval for the ratio of alleles. All statistical probabilities used in the study were conducted with 95% confidence (95% CI).

Table 1. Primer pairs were used in this study.

Primer	5' sequence	3' sequence	Size of products
ALF1	GCTGACATCATCCCCAACT	CTGGAATGTGCTATGGTG	1000 bp
ALF2	ACTAACCCTTTCGCTCCCACCCAC	TATTGGATGAGGTATCAACATTCGC	750 bp
AIF	TCTAGGGGAGTAGGAGGAATCA	GTGGACCACTGAGAAGGTCA	580 bp
BGB	TACGGCTGCTCCCGAACT	TACGAGGCAACATCGAAATA	560 bp
CAL	TAGAGATCGTATTACGTCAAAGGA	GGTCAAGAACTCGTGGAACACCT	250 bp
HAE	GCAGATTCCGAGCATTTACGC	TAGAACACTTTGAAACTGCCACC	750 bp
P53	AGTCGTGTTTTTTAGTCTTAAGTC	CAGGAAGTGGTAGAGCCTTCAATCA	1500 bp
RAB5B	GGAGACCTCTGCTAAGACTGCTATG	CTGTGCTGGCTGGTTATTGG	350 bp
TRAF6	CTGACCCTTTAGTGGACGCAT	AGGTTCCTGTGCTGGGTTGA	250 bp

RESULTS AND DISCUSSION

In this study, nine primer pairs have been used to amplify SNPs in shrimp samples. PCR products were electrophoresed on 1% agarose gel. Results on Fig. 1 showed that a single band with the expected size in all the samples (750 bp band for *HAE* gene, 1500 bp band for *P53* gene, and 250 bp band for *TRAF6* gene, respectively).

The PCR products were purified and sequenced directly in ABI PRISM 3500 system. The data were compared with reference genes of *L. vannamei* in GenBank. The comparison results of gene sequences at SNP positions were shown in Fig. 2, 3, 4, 5.

Allele frequencies of SNP loci were recorded and calculated in each group of shrimp samples. Results on Table 2 showed that six SNPs (in *AIF*, *ALF1*, *HAE*, *P53*, *Rab5B*, and *TRAF6* genes) were in accordance with Hardy–Weinberg Equilibrium (HWE) ($p > 0.05$) while three SNPs (in *ALF2*, *BGB*, and *CAL*) were not ($p < 0.05$).

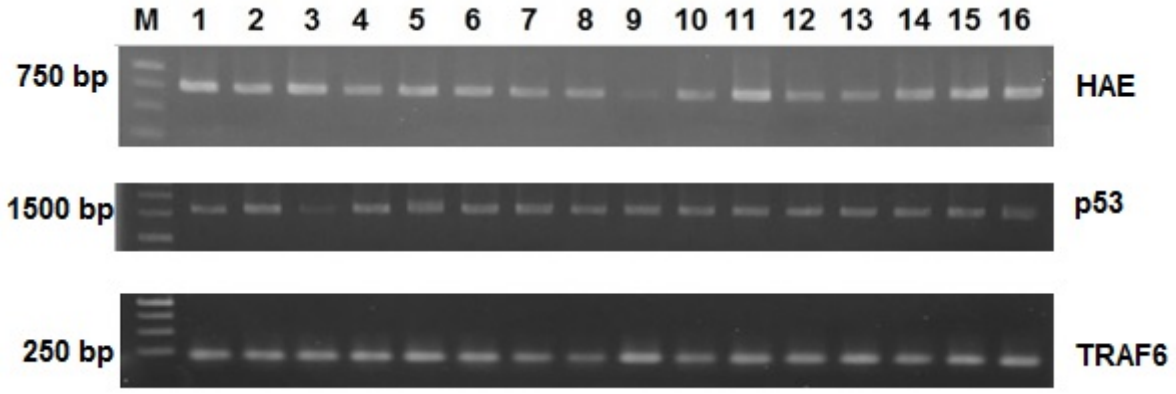

Figure 1. Electrophoresis results of PCR products on 1% agarose gel. (First section: Amplified PCR products of *HAE* gene from shrimp samples**.** Second section: Amplified PCR products of *p53* gene from shrimp samples**.** Third section: Amplified PCR products of *HAE* gene from shrimp samples).

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Query  440  TTCAACTCCAGTGTTGAGAGTTAACACCACTTTGCCATCATCATTGGTGCTGACGCCCTT  499
            ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||| |||
Sbjct  811  TTCAACTCCAGTGTTGAGAGTTAACACCACTTTGCCATCATCATTGGTACTGACGCTCTT  752

Query  500  AACATAGGAGTTGGGGATGATGTCAGCTCCTTCTGACTTGACCTTCTCAGTGGGTCCAC  558
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  751  AACATAGGAGTTGGGGATGATGTCAGCTCCTTCTGACTTGACCTTCTCAGTGG-TCCAC  694
```

Figure 2. Comparison results with the cDNA sequence of *AIF* gene (*L. vannamei* apoptosis inducing factor – KX096891) SNP locates at position 601 on cDNA sequence of *AIF* gene.

```
Query  27    CACCAGCGGGTAGGAGGCCTGGCTGGCGATGCCGCAGTTGTTGTTCCTGTTTCGGGACAT 86
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1793  CACCAGCGGGTAGGAGGCCTGGCTGGCGATGCCGCAGTTGTTGTTCCTGTTTCGGGACAT  1734
              ↓
Query  87    CTTGATGTATCCCTTGTCGCCCCAGGAGGTGTTCCACGAGTTCTTGACCA  136
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1733  TTTGATGTATCCCTTGTCGCCCCAGGAGGTGTTCCACGAGTTCTTGACCA  1684
```

Figure 3. Comparíon results with the cDNA sequence of cathepsin L gene (*L. vannamei* cathepsin L – Y13924). SNP locates at position 212 on cDNA sequence of *CAL* gene.

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Query  305   TGATACCATTGTTGTCGAGGTGAGGCCAGGCGAAAATGCGCACGGTAGCTAGAACTTCCT  364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1543  TGATACCATTGTTGTCGAGGTGAGGCCAGGCGAAAATGCGCACGGTAGCTAGAACTTCCT  1484
                                  ↓
Query  365   CGCCCTTGTTATTGCTAACATCAACTTTGATTTTAAAGTCCTTGTGGTTGAGACGAGGAA  424
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  1483  CGCCCTTGTTATTGCTAATATCAACTTTGATTTTAAAGTCCTTGTGGTTGAGACGAGGAA  1424
```

Figure 4. Comparíon results with the cDNA sequence of *HAE* gene (*L. vannamei* hemocyanin subunit L1 – KF193058). SNP locates at position 1465 on cDNA sequence of *HAE* gene.

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              ↓
Query  3037  CAACAGCAAAGAAACTGCCAAAGAGTGACAGTACTGCAAGCGGTTCTCCTAGGGGTGATG  3096
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  718   CAATAGCAAAGAAACTGCCAAAGAGTGACAGTACTGCAAGCGGTTCTCCTAGGGGTGATG  777

Query  3097  TCAGCCTGTCCAATAACCAGCCAGCACAGGGCACTGCTGGATGTTGCAAGTG  3148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  778   TCAGCCTGTCCAATAACCAGCCAGCACAGGGCACTGCTGGATGTTGCAAGTG  829
```

Figure 5. Comparíon results with the cDNA sequence of *Rab5B* gene (*L. vannamei* Rab5B – JQ901103). SNP locates at position 524 on cDNA sequence of *Rab5B* gene.

In this study, we assessed the correlation between genotype and phenotype of some SNPs in genes involved in WSSV resistance in shrimp, *L. vannamei*. The results indicated that no significant difference in allele frequencies between disease and resistance group was found in *P53* and *TRAF6* genes in all three tested models (p>0.05) (the data not showed). In Table 3 showed a significant difference of genotypes obtained in the additive model of four genes (*AIF*, *ALF1*, *HAE*, and *Rab5B* with p = 0.000). For *AIF* and *ALF1* genes, the frequencies of GG and CC genotypes were significantly different between two groups and were associated with a decreased resistance with WSSV compared to the AA and TT genotypes (p = 0.021 and p = 0.017, respectively). The G and C alleles were associated with a decreased resistance with WSSV (OR = 0.184; 95% CI: 0.074–0.456; p = 0.000 and OR = 0.229; 95% CI: 0.096–0.545; p = 0.001, respectively). For *HAE* gene, the frequency of TT genotype was significantly different between two groups and was associated with a increased resistance with WSSV compared to the TC+CC genotype (OR = 68.750; 95% CI: 11.462–412.381; p = 0.000). For *Rab5B*

gene, the frequency of CC genotype was significantly different between two groups and was associated with an increased resistance with WSSV compared to the TT genotype in all three tested models (OR = 14.400, 95% CI: 2.714–118.109, p = 0.004; OR = 4.800, 95% CI: 1.372–16.795, p = 0.011; and OR = 7.765, 95% CI: 1.529–39.442, p = 0.007, respectively). The C allele was associated with an increased resistance with WSSV (OR = 3.974; 95% CI: 1.754–9.003; p = 0.001).

Table 2. General information on the studied single nucleotide polymorphisms (SNPs).

Gene	Allele	Type of variant	HWE in disease group (p)	HWE in resistance group (p)	HWE in all population (*p*)
ALF1	T/C	p.Phe51Phe	0.02	0.09	0.54
ALF2	G/T	p.Glu54Asn	0.02	0.01	0.00
AIF	A/G	p.Ser201Gly	0.41	0.00	0.07
BGB	T/C	p.Gly158Gly	0.39	0.01	0.00
CAL	T/C	p.Phe71Ser	0.74	0.00	0.00
HAE	A/G	p.Ile489Val	0.00	0.00	0.87
Rab5B	T/C	p.Ile175His	0.97	0.51	0.22
P53	-/A	5'UTR	0.61	0.26	0.26
TRAF6	A/G	p.Ile500Val	0.09	0.66	0.15

HWE: Hardy-Weinberg equilibrium was checked by Chi-squared test.

Table 3. Associations of SNPs with WSSV resistance.

Gene	Test model	OR	95% CI	*P*	Gene	Test model	OR	95% CI	*P*
AIF	**Additive**			**0.000**	***HAE***	**Additive**			**0.000**
	AA	1.00				AA	1.00		
	AG	0.048	0.008 – 0.201	**0.000**		AG	0.000		0.997
	GG	0.130	0.020 – 0.697	**0.021**		GG	0.000		0.998
	Dominant					**Dominant**			
	AA	1.00				AA	1.00		
	AG+GG	0.069	0.018 – 0.026	**0.000**		AG+GG	1.302	0.312 – 5.436	0.717
	Recessive					**Recessive**			
	AG+AA	1.00				AG+AA	1.00		
	GG	0.438	0.093 – 2.063	0.288		GG	68.750	11.462 – 412.381	**0.000**
	Allele					**Allele**			
	A	1.00				A	1.00		
	G	0.018	0.074 – 0.456	**0.000**		G	0.189	0.074 – 0.483	**0.000**
ALF1	**Additive**			**0.000**	***Rab5B***	**Additive**			**0.007**
	TT	1.00				TT	1.00		
	TC	2.375	0.380 – 14.126	0.334		TC	2.880	0.781 – 11.757	0.122
	CC	0.118	0.017 – 0.634	**0.017**		CC	14.400	2.714 – 118.109	**0.004**
	Dominant					**Dominant**			
	TT	1.00				TT	1.00		
	TC+CC	0.548	0.121 – 2.471	0.429		TC+CC	4.800	1.372 – 16.795	**0.011**
	Recessive					**Recessive**			
	TC+TT	1.00				TC+TT	1.00		
	CC	0.066	0.017 – 0.260	**0.000**		CC	7.765	1.529 – 39.442	**0.007**
	Allele					**Allele**			
	T	1.00				T	1.00		
	C	0.229	0.096 – 0.545	**0.000**		C	3.974	1.754 – 9.003	**0.001**

95% CI: 95% confidence interval of odds ratio; OR: Odds ratio.

The above evaluation results suggested that the potential SNPs of these *AIF*, *ALF1*, *HAE*, and *Rab5B* genes can be used as the molecular markers for breeding selection the resistance to white spot disease in white-legged shrimp *L. vannamei*.

CONCLUSION

In this study, we assessed the correlation between genotype and phenotype of some SNPs in genes involved in WSSV resistance in shrimp, *L. vannamei*. Six SNPs (in *AIF*, *ALF1*, *HAE*, *P53*, *Rab5B*, and *TRAF6* genes) were in accordance with Hardy–Weinberg Equilibrium (HWE) ($p > 0.05$) while three SNPs (in *ALF2*, *BGB*, and *CAL*) were not ($p < 0.05$). Four SNPs in *AIF*, *ALF1*, *HAE*, and *Rab5B* genes had significant differences of genotypes between disease and resistance groups. The evaluation results suggested that the potential SNPs of these *AIF*, *ALF1*, *HAE*, and *Rab5B* genes can be used as the molecular markers for breeding selection the resistance to white spot disease in white-legged shrimp *L. vannamei*.

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Abbreviation

AIF: apoptosis inducing factor

ALF1: anti-lipopolysaccharide factor

AMPs: antimicrobial peptide

BGB: β-1,3-glucan-binding protein

CAL: cathepsin L

CAR: carboxypeptidase

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CYC: cytochrome c

DNA: deoxyribonucleotides acid

GTPase: guanosine triphosphatase

HAE: haemocyanin

HSP: heat shock protein

IAP: inhibitor of apoptosis protein

IE: immediate-early

IMD: immune deficiency

PCR: polymerase chain reaction

SNP: single nucleotide polymorphism

SPSS: statistical package for the social sciences

TRAF6: TNF receptor associated factor 6

VEGF: vascular endothelial growth factor

WSSV: white spot syndrome virus

ĐÁNH GIÁ CÁC CHỈ THỊ SNP TIỀM NĂNG CHO CHỌN GIỐNG KHÁNG VIRUS GÂY BỆNH ĐỐM TRẮNG Ở TÔM THẺ CHÂN TRẮNG (*LITOPENAEUS VANNAMEI*)

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TÓM TẮT

WSSV (virus gây bệnh đốm trắng) là một loại virus nguy hiểm và là nguyên nhân gây thiệt hại nghiêm trọng cho nghề nuôi tôm. Nhiều gen liên quan đến các cơ chế kháng bệnh đã được xác định và nghiên cứu. Trong nghiên cứu này, chúng tôi đã khuếch đại và giải trình tự 9 đoạn gen mang các điểm SNPs trên các gen liên quan đến tính kháng bệnh đốm trắng trên tôm thẻ chân trắng, *L. vannamei*. Tần suất alen tại các điểm SNPs được ghi nhận và tính toán bằng phần mềm thống kê sinh học SPSS (version 22) ở các nhóm tôm nghiên cứu: nhóm tôm bị bệnh (được cảm nhiễm nhân tạo với WSSV và chết) và nhóm tôm kháng bệnh (được cảm nhiễm nhân tạo với WSSV nhưng vẫn khỏe mạnh). Sáu điểm SNP trên các gen *AIF*, *ALF1*, *HAE*, *P53*, *Rab5B*, và *TRAF6* phù hợp với trạng thái cân bằng Hardy-Weinberg (HWE) ($p>0,05$) và ba SNP trên các gen *ALF2*, *BGB* và *CAL* không phù hợp ($p<0,05$). Kết quả đánh giá trên gen *AIF* và *ALF1* cho thấy có sự khác biệt có ý nghĩa thống kê ($p < 0,05$) về tần suất kiểu gen GG và CC giữa nhóm tôm bệnh và nhóm tôm kháng. Các alen G và C có liên quan đến sự giảm tính kháng với WSSV ($p = 0.000$ và $p = 0.001$, tương ứng). Tần suất kiểu gen TT giữa nhóm tôm kháng và nhóm tôm bệnh cũng có sự khác biệt có ý nghĩa thống kê ($p < 0,05$) ở điểm SNP trên gen *HAE* so với kiểu gen TC+CC. Đặc biệt ở điểm SNP trên gen *Rab5B* cho thấy sự khác biệt và sự gia tăng tính kháng với bệnh một cách có ý nghĩa thống kê ($p < 0,05$) của tần suất kiểu gen CC giữa nhóm tôm kháng và nhóm tôm bệnh so với kiểu gen TT ở cả ba mô hình đánh giá. Các kết quả đánh giá trên chỉ ra rằng có thể sử dụng các chỉ thị trên gen *AIF*, *ALF1*, *HAE và Rab5B* trong chọn giống kháng bệnh đốm trắng ở tôm thẻ chân trắng *L. vannamei*.

Từ khóa: Chỉ thị phân tử, tính kháng WSSV, *Litopenaeus vannamei*, SNP