

The polymorphisms of chemokine gene in channel catfish (*Ictalurus punctatus*) and the associations with susceptibility/resistance to *Edwardsiella ictaluri*

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Abstract

Chemokines play a crucial role in the recruitment, activation and adhesion of immune effector cells to the foci of infection and injury under both homeostatic and inflammatory conditions. Identification of markers of chemokine genes associated with resistance to pathogens is necessary for the selective breeding of channel catfish. In the present study, the polymorphisms of genomic sequence of a CC chemokine, SCYA-115, were investigated in susceptible and resistant groups of channel catfish (*Ictalurus punctatus*). The polymorphisms in putative binding sites for transcription factor in promoter region were also investigated to explore their associations with susceptibility/resistance to *Edwardsiella ictaluri*. Thirty-five sites of SNPs and nine sites of ins-del polymorphisms were discovered in the complete sequence of SCYA-115. Among them, five SNP sites and one ins-del polymorphisms were found in the region of binding sites for the transcription factor. The allele and genotype distributions were examined at these six polymorphisms. The results indicate that -820 T allele, -752 ins allele and -752 ins/del genotype were more prevalent in resistant group than that in susceptible group, which suggested that the carriers of these polymorphisms are more resistant to *E. ictaluri*. In addition, significant differences were found in haplotypes between susceptible and resistant groups. The results provide candidate markers for the selection of channel catfish with enhanced resistance to *E. ictaluri*.

Keywords: Chemokine, polymorphism, channel catfish *ictalurus punctatus*, disease resistance, disease susceptibility

Background

Chemokines, the superfamily of chemotactic cytokine, are small proteins produced by infected tissues in the early stages of infection [1,2]. They are responsible for the recruitment, activation and adhesion of various leukocytes to inflammatory foci under both homeostatic and inflammatory conditions [3,4]. They are considered to be necessary to translate an innate immune response into adaptive response, serving as a bridge between innate and adaptive immunity [5]. Chemokines are divided into four subfamilies C, CC, CXC and CX3C based on the arrangement of the first two conserved cysteine residues, which exist in the majority of chemokine structures [6,7]. CC chemokines, distinguished by adjacent cysteine residues in a conserved position, constitute the largest subfamily of chemokines with 24 CC chemokines identified from humans [3,6]. In teleost fish, researchers have identified 26, 18, 30, and 81 CC chemokines in channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and zebrafish (*Danio rerio*), respectively [8-13]. Due to the diverse immune roles of CC chemokines, seven groups of

CC chemokines were established in teleost fish through phylogenetic analysis and were named the CCL20 group, CCL27/28 group, MCP group, MIP group, CCL17/22 group, CCL19/21/25 group, and the Fish (specific) CC group [9].

CC chemokine polymorphisms, perhaps affecting the quality or quantity of these genes, are considered as the potential candidates for markers associated with susceptibility/resistance to pathogens, and may change the immune capacity of individuals to protect itself against infection [14]. In human, many studies have shown that chemokine polymorphisms influence the immune response and disease resistance [15,16]. For example, -403 A allele in promoter region of RANTES, a CC chemokine, is suggested to be a risk factor for HIV transmission and a protective factor for HIV progression [17]. Although advances have been achieved in the study of the identification of chemokine genes in teleost fish, there is very limited information in terms of the associations between chemokine polymorphisms and susceptibility/resistance to pathogenic organisms. Baoprasertkul *et al.*, have found 14 types of cDNA sequences from a single

F1 hybrid catfish (channel catfish × blue catfish), whereas its association with susceptibility/resistance to pathogens was not known [48].

Channel catfish is one of the most important aquaculture species worldwide, accounting for approximately 444,937 tons of the production and 675,342,000 dollars of the profit, respectively [18]. Nevertheless, catfish production suffered massive financial losses due to pathogen spread and breakouts such as enteric septicemia of catfish (ESC), caused by a Gram-negative intracellular bacterium *Edwardsiella ictaluri* [2]. Given that CC chemokines play an important role in innate immunity, it is worthy to investigate the associations of polymorphisms of CC chemokines with the susceptibility/resistance to ESC. In the present study, the sequence of CC chemokine SCYA-115 gene (hereinafter referred to as SCYA-115), the expression of which was significantly upregulated upon bacterial infection, was employed from GenBank to amplify the corresponding genomic sequence in channel catfish [19]. The aim of this work was to (a) identify the polymorphisms of SCYA-115 genomic sequence; (b) determine the variation of putative binding sites for transcription factor; (c) test the relation between the polymorphisms and susceptibility/resistance to ESC. This research will provide potential markers and contribute greatly to selective breeding of resistant channel catfish to ESC.

Materials and Methods

Animals and DNA extraction

All experimental fish, 2-3-year-old channel catfish, were collected from catfish farms (Hubei province in China). The fish selected for resistance to ESC for three generations were categorized as resistant group, and the fish random sampled without selective breeding were categorized as susceptible group. When exposed to *E. ictaluri*, the survival rate of fish in resistant group was significantly higher than that in susceptible group (data not shown). The muscle tissues of each fish were removed and kept at -20°C until DNA isolation.

About 100 mg muscle tissues of each fish was homogenized in 500 mL buffer containing 100 mmol L⁻¹ EDTA, 10 mmol L⁻¹ Tris-HCl, 1% SDS and 0.1 mg mL⁻¹ proteinase K. The mixture was incubated at 55°C for 2.5 h. And then, DNA was extracted with phenol, phenol/chloroform (1:1), and chloroform/isoamyl alcohol (24:1), and precipitated with two volumes of ice-chilled absolute ethanol. After washing twice with 75% ethanol, the DNA pellet was dried and resuspended in sterile water with a concentration of 50 ng/μL, and stored in -20°C.

Identification of polymorphic loci in SCYA-115

A pair of gene specific primers, SCYA-115p1 (5'-AGGGAATGGGTTGTTT-3') and SCYA-115p2 (5'-CTGTTTTATTGTCGTAT-3') was designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) based on the

sequence of SCYA-115 (GenBank accession No. DQ173289). They were used to amplify a 3496-bp fragment of the gene sequence. PCR reaction was performed in a thermal cycler (Applied Biosystems) in 50 μL reaction volume containing 2.5ng of DNA template, 2 μL of each primer (10 μmol L⁻¹), 5 μL of 10' LA PCR buffer (Mg²⁺ Plus), 8 μL of dNTP Mixture (2.5 mmol L⁻¹), 30.5 μL of sterile water and 0.5μL (5U μL⁻¹) of *LA Taq* polymerase (TakaRa). The PCR temperature profile was as follows: 95°C for 5 min; 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 3.5 min; a further 8 min elongation at 72°C.

The PCR products were detected by electrophoresis on 1% agarose gels and the fragments were excised and purified. The objective fragments were then cloned into PMD18-T vector, transformed into *Escherichia coli* JM109, and three clones were sequenced for each fragment using an ABI PRISM 3500 DNA Analyzer. The sequence alignments of SCYA-115 were performed using program MEGA 4.0 and polymorphisms were detected from the alignments of different individuals.

Association analysis between polymorphisms and susceptibility/resistance to ESC

Twenty-five more individuals of each susceptible and resistant group were sampled and DNA was extracted for the analysis of the associations between polymorphisms and susceptibility/resistance. Another pair of primers, SCYA-115p3 (5'-AGGGAATGGGTTGTTTC-3') and SCYA-115p4 (5'-TGAGCGGAGGATTGG-3') was designed to amplify a 510-bp fragment of the promoter sequence, which was showed to have SNP or ins/del in the putative binding sites for transcription factor. The PCR reaction and DNA sequence analysis were performed as described above.

Statistical analysis

The promoter region of SCYA-115 was analyzed by TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>) to predict putative binding sites for transcription factor located at the polymorphisms [14]. The predicted amino acid sequences of SCYA-115 were analyzed using Primer Premier 5.0 software. RepeatMasker (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>) was used to determine the presence and types of repetitive sequences in SCYA-115 genomic DNA sequence [20]. The allele and genotype frequencies were determined by direct counting. SHEsis (<http://analysis.bio-x.cn>) was used to estimate haplotype frequency and linkage disequilibrium. The associations between the polymorphisms and susceptibility/resistance to ESC were analyzed with chi-square test (χ^2 test) in SPSS 17.0 software (SPSS, Inc., Chicago, IL). Further analysis was performed, for the polymorphism loci having significant associations with the susceptibility/resistance to ESC, using a stepwise binary logistic regression to determine the risk/protection factor. The quality of genotype data for each polymorphism was determined by testing for Hardy-Weinberg equilibrium using HWE program [49]. *P* value less

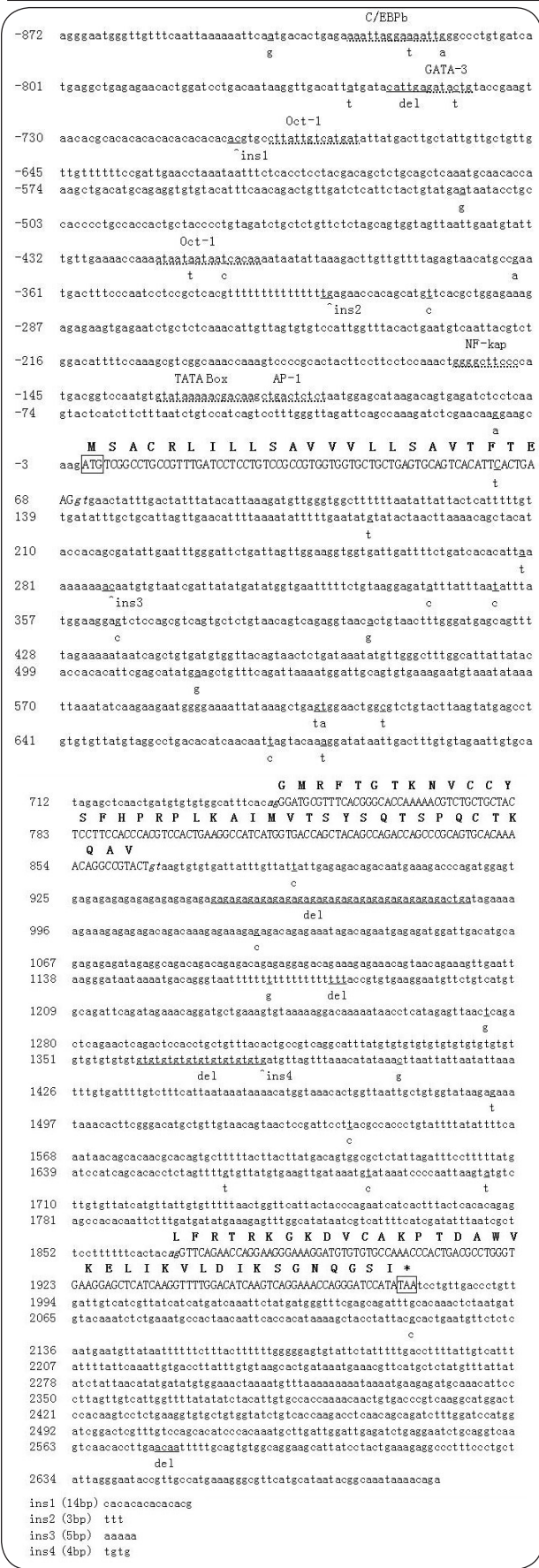


Figure 1. Polymorphisms and immune-related binding sites for transcription factor of SCYA-115 in channel catfish

The nucleotides are numbered on the left. Exons are indicated in capital letters while introns are in small letters. The start and stop codons are boxed. The intron 5' GT and 3' AG are in italic letters. The deduced amino acid sequence is shown in bold lettering. Several of putative transcription factor binding sites are indicated by dots line. The polymorphism factor are underlined and the variants are described below.

Table 1. Repetitive elements in the genomic sequence of SCYA-115.

score	diverged bp (%)	deleted bp (%)	inserted bp (%)	begin	end	repeat	Class/family
213	3.9	0.0	0.0	144	183	(CA) _n	Simple_repeat
318	16.0	1.0	0.0	1766	1865	L1-55_XT	LINE/L1
593	19.4	0.5	0.0	1782	2003	IMPB_01	Satellite
29	48.3	0.0	0.0	2030	2058	AT_rich	Low_complexity
486	0.0	0.0	0.0	2201	2258	(TG) _n	Simple_repeat
22	62.1	0.0	0.0	2273	2301	AT_rich	Low_complexity
283	24.2	0.8	0.7	3346	3450	Tc1-4Ory	DNA/TcMar-Tc1

The scores were derived from RepeatMasker.

than 0.05 was considered statistically significant.

Results

The polymorphisms of genomic DNA sequence and repetitive sequences scan

Thirty-five sites of SNPs and nine sites of ins-del polymorphisms were identified in SCYA-115 by the alignments of genomic DNA sequence from different individuals (Figure 1). They were deposited in GenBank with accession No. KC533705-KC533714. Among the polymorphisms detected, only one synonymous SNP, C to T transition, was found in exon.

Several types of repetitive sequences were found in the SCYA-115 genomic sequence (Table 1). One simple repeat located in promoter region. Several consensus elements of binding sites for transcription factor were identified in the promoter region of SCYA-115, including C/EBPb, Oct-1, NF-kap and AP-1. These sites were responsible for regulation of immune reactions, suggesting the role of SCYA-115 in immune response. Moreover, classical TATA box and GATA were also found in the promoter region.

Polymorphisms in promoter region of SCYA-115

Eleven SNP sites and three ins-del polymorphisms were found to locate in promoter region. With screening the putative binding sites for transcription factor by TFSEARCH analysis, five SNP sites and one ins-del polymorphisms were found in the region of binding sites (Figure 2). At locus -820, a putative binding site for C/EBPb was found in A allele, while putative binding sites for GATA-1, GATA-2, S8 and Nkx-2 were found in T allele. At locus -752 and -742, there were putative binding sites for GATA-1, GATA-2 and GATA-3 in ins-C haplotype, and GATA-2 and GATA-3 in ins-T haplotype, but no binding sites for

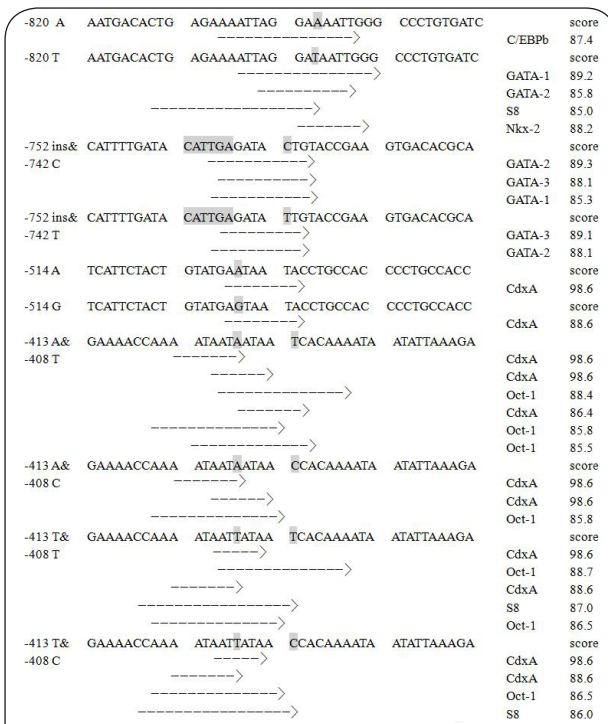


Figure 2. Putative binding sites for transcription factor between different alleles or haplotype
 Polymorphic loci are shadowed. The positions and directions of putative binding sites for transcription factor are labeled by arrowhead. The scores from TFSEARCH analysis are shown on the right.

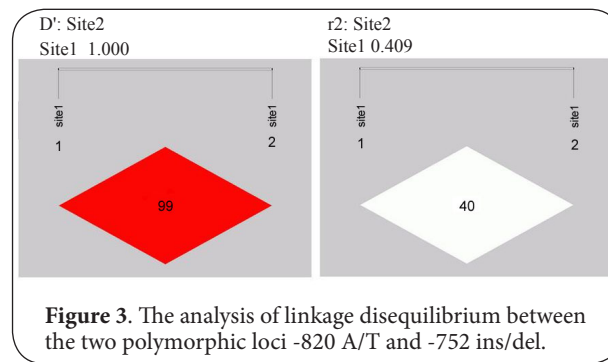


Figure 3. The analysis of linkage disequilibrium between the two polymorphic loci -820 A/T and -752 ins/del.

transcription factor was found in del-C or del-T haplotype. At locus -514, the putative binding site for CdxA was found in both A allele and G allele. At locus -413 and -408, there were putative binding sites for CdxA and Oct-1 in both A-T haplotype and A-C haplotype, and CdxA, Oct-1 and S8 in both T-T haplotype and T-C haplotype.

The associations between polymorphisms in promoter region and susceptibility/resistance to ESC

The associations between polymorphisms in promoter region and susceptibility/resistance to ESC was investigated by examining the distributions of polymorphisms in six loci (-820 A-T SNP, -752 ins-del, -742 C-T SNP, -514 A-G SNP,

-413 A-T SNP and -408 C-T SNP) of susceptible and resistant groups. With the analysis of HWE, the genotypic frequency of allele was demonstrated to be in the Hardy-Weinberg equilibrium ($P > 0.05$) at each of the six loci (Table 2).

At locus-820, the T allele frequency was 51.67% in susceptible group, while 70.00% in resistant group (Table 2). χ^2 -test showed a significant difference in its frequency distribution between the two groups ($P = 0.040$). While no significant difference was found among the frequency of three genotypes, including A/A, A/T and T/T. The locus of -820 A/T was proved to be significantly associated with the susceptibility/resistance to ESC in allele analysis, and T allele was suggested to be the protection factor on resistance to ESC through the analysis of logistic regression (OR=2.18, B=0.78, $P = 0.041$ for A allele).

At locus -752, the ins allele frequency was 13.33% in susceptible group, while 28.33% in resistant group. The ins/del genotype frequency was 20.00% in susceptible group, while 50.00% in resistant group (Table 2). χ^2 -test revealed a significant difference in the frequency distribution of both allele and genotype between the two groups ($P = 0.043$ for allele frequency; $P = 0.049$ for genotype frequency). The locus of -752 ins/del was proved to be significantly associated with the susceptibility/resistance to ESC in both allele and genotype analysis. Ins allele and ins/del genotype were suggested to be the protection factors on resistance to ESC (OR=0.39, B=-0.94, $P = 0.047$ for ins allele; B=-1.41, $P = 0.017$ for ins/del genotype).

The rest of polymorphic loci, including -742 C/T, -514 A/G, -413A/T and -408 C/T, had no significant difference in the frequency distribution of neither allele nor genotype between susceptible and resistant groups (Table 2).

In addition, the statistical analysis strongly suggested the presence of linkage disequilibrium between the two polymorphic loci -820 A/T and -752 ins/del ($D = 1.000$, $r^2 = 0.409$) (Figure 3). The haplotype of T-ins was even not observed in the present study. With the analysis performed at haplotype level, significant differences were found in haplotypes between susceptible and resistant groups (Table 3). The A-del haplotype was suggested to be the risk factor with OR as high as 31.77.

Discussion

In the recent years, the aquaculture industry of catfish has suffered massive financial losses due to the spread and breakouts of ESC. The selective breeding for disease-resistant strains is regarded as an effective approach contributing to an increase in survival during pathogen challenge. Molecular markers in the immune-related genes, such as MHC class IB, MHC class IIB, TLR3, TLR22, Mx2 and MDA5, have been extensively identified in teleost fish, and served as an important role in selective breeding [21-26]. For example, the polymorphisms of MBL in zebrafish were demonstrated to be associated with susceptibility/resistance to *Listonella anguillarum* [27]. Possible associations of particular MDA5

Table 2. The frequency distributions of SCYA-115 gene polymorphisms in binding sites for transcription factor in susceptible and resistant groups.

Polymorphism locus	Susceptible individuals NO. (%)	Resistant individuals NO. (%)	χ^2 (P)	OR (95% CI)	B (P)	Wald
-820	-	-	-	-	-	-
Allele						
A	29 (48.33)	18 (30.00)	4.23 (0.040*)	2.18	0.78 (0.041)	4.17
T	31 (51.67)	42 (70.00)	-	(1.03-4.62)	-	-
Genotype						
A/A	5 (16.67)	1 (3.33)	5.50 (0.064)	-	-	-
A/T	19 (63.33)	16 (53.33)	-	-	-	-
T/T	6 (20.00)	13 (43.33)	-	-	-	-
HWE χ^2 (P)	2.16 (0.14)	2.18 (0.14)	-	-	-	-
-752	-	-	-	-	-	-
Allele						
ins	8 (13.33)	17 (28.33)	4.09 (0.043)	0.39	-0.94 (0.047)	3.94
del	52 (86.67)	43 (71.67)	-	(0.15-0.99)	-	-
Genotype						
ins/ins	1 (3.33)	1 (3.33)	6.05 (0.049)	-	-0.50 (0.73)	0.12
ins/del	6 (20.00)	15 (50.00)	-	-	-1.41 (0.017)	5.73
del/del	23 (76.67)	14 (46.67)	-	-	-	-
HWE χ^2 (P)	0.54 (0.46)	1.60 (0.21)	-	-	-	-
-742	-	-	-	-	-	-
Allele						
C	55 (91.67)	56 (93.33)	0.120 (0.73)	0.79	-	-
T	5 (8.33)	4 (6.67)	-	(0.20-3.08)	-	-
Genotype						
C/C	25 (83.33)	26 (86.67)	0.131 (0.72)	0.77	-	-
C/T	5 (16.67)	4 (13.33)	-	(0.19-3.20)	-	-
HWE χ^2 (P)	0.25 (0.62)	0.15 (0.70)	-	-	-	-
-514	-	-	-	-	-	-
Allele						
A	53 (88.33)	51 (85.00)	0.288 (0.59)	1.34	-	-
G	7 (11.67)	9 (15.00)	-	(0.46-3.86)	-	-
Genotype						
A/A	23 (76.67)	21 (70.00)	0.341 (0.56)	1.41	-	-
A/G	7 (23.33)	9 (30.00)	-	(0.45-4.45)	-	-
HWE χ^2 (P)	0.52 (0.47)	0.93 (0.33)	-	-	-	-
-413	-	-	-	-	-	-
Allele						
A	43 (71.67)	39 (65.00)	0.62 (0.43)	1.36	-	-
T	17 (28.33)	21 (35.00)	-	(0.63-2.95)	-	-
Genotype						
A/A	15 (50.00)	11 (36.67)	1.15 (0.56)	-	-	-
A/T	13 (43.33)	17 (56.67)	-	-	-	-
T/T	2 (6.67)	2 (6.67)	-	-	-	-
HWE χ^2 (P)	0.14 (0.71)	1.81 (0.18)	-	-	-	-
-408	-	-	-	-	-	-
Allele						
C	3 (5.00)	4 (6.67)	0.15 (0.70)	0.74	-	-
T	57 (95.00)	56 (93.33)	-	(0.16-3.44)	-	-
Genotype						
C/T	3 (10.00)	4 (13.33)	0.16 (0.69)	0.72	-	-
T/T	27 (90.00)	26 (86.67)	-	(0.15-3.55)	-	-
HWE χ^2 (P)	0.083 (0.77)	0.15 (0.70)	-	-	-	-

OR= odds ratio; B indicated partial regression coefficient in logistic regression

*The distributions in susceptible and resistant groups were significantly different (P<0.05).

alleles and genotypes with susceptibility/resistance to grass carp reovirus were reported in grass carp [23]. However, there is very limited information in terms of the polymorphisms

of immune-related genes in catfish.

In the present study, SCYA-115 chemokine was chosen as the candidate gene for analysis. SCYA-115 was demonstrated

Table 3. Haplotype distribution of -820 A/T and -752 ins/del in susceptible and resistant groups.

Haplotype	Haplotype frequency %		Chi2	Pearson's P	OR (95% CI)
	Susceptible individuals	Resistant individuals			
A ins	13.33	28.33	4.093	0.043*	0.39 (0.15-0.99)
A del	35.00	1.67	22.26	0.0000024	31.77 (4.10-245.91)
T del	51.67	70.00	4.23	0.040	0.46 (0.22-0.97)

OR= odds ratio.

*The distributions in susceptible and resistant groups were significantly different ($P < 0.05$).

to be the one of seven chemokines, the expression levels of which were significantly upregulated upon ESC infection, and the promoter region of SCYA-115 has been better sequenced for subsequent analysis [19]. Thirty-five sites of SNPs and nine sites of ins-del polymorphisms were found in the genomic sequence, and five SNP sites and one ins-del polymorphisms of them located in the region of putative binding sites for transcription factor. Results from statistical analysis suggested that -820 T allele was more prevalent in resistant group than that in susceptible group ($P = 0.040$), which indicated that -820 T carrier would be more resistant and -820 T could be a genetic protection factor in ESC infection. At locus -752, both ins allele and ins/del genotype were showed to be more prevalent in resistant group than that in susceptible group ($P = 0.043$; $P = 0.049$). It could be expected that -752 ins allele and ins/del genotype are also protection factors in ESC infection. Major progresses of recent studies in the chemokine polymorphisms are concentrated in human, while the explorations in teleost fish have only just begun [17,28-32]. To our knowledge, this is the first study demonstrating the associations between the chemokine polymorphisms and susceptibility/resistance to pathogens in catfish.

Polymorphisms in promoter region of various genes have been extensively demonstrated to be associated with disease resistance in some species [33-36]. In the present study, two polymorphic loci, including -820 T allele, -752 ins allele and -752 ins/del genotype, were identified to be significantly associated with disease resistance, probably due to the effects on transcription and splicing process [37-39]. In -820 T allele, four extra putative binding sites for transcription factor, including GATA-1, GATA-2, S8 and Nkx-2, were detected, while carrying T allele resulted in abolition of the binding site C/EBPb. In -752 ins allele, three members of GATA family, including GATA-1, GATA-2 and GATA-3, were detected compared to del allele. GATA zinc-finger transcription factor, a positive cis-regulatory element, was traditionally thought to play critical roles in development and differentiation in both vertebrates and invertebrates. However, accumulating evidences have suggested its crucial roles in regulating immune responses in recent years. GATA factors were suggested to be responsible for regulating a systemic induction of antimicrobial peptides in *Drosophila* in response to systemic infection [40-43].

The roles of Nkx-2 and C/EBPb in immune responses have also been identified, and C/EBP is demonstrated to influence both basal and inducible activities of the Interleukin-8 promoter in response to stimulation with IL-1 β [44-46]. Consequently, the extra putative binding sites for transcription factor might affect the transcription of SCYA-115, and consequently influence the susceptibility/resistance phenotype of channel catfish to ESC infection. In addition, the strong linkage disequilibrium between the two polymorphic loci -820 A/T and -752 ins/del might be another reason for the associations between both loci and the susceptibility/resistance to ESC. However, the missing of binding site C/EBPb could not explain the elevated resistance to ESC, and further studies are needed.

In both susceptible and resistant groups, no T-ins haplotype (-820&-752) was observed, which suggested that T-ins haplotype might have been eliminated by natural selection for disruption the crucial binding site or affect splice process [23]. Furthermore, increasing evidences showed that intronic genetic variations even a single point mutation can cause deleterious implications for gene splicing [23,47]. Further work is warranted to screen the associations between polymorphisms in intron and susceptibility/resistance to pathogens, and investigate the possible mechanisms of all these associations.

Conclusions

In summary, polymorphisms of SCYA-115 genomic sequence, especially in putative binding sites for transcription factor, were investigated, and significant associations between two polymorphism loci and disease susceptibility/resistance were established in channel catfish. The results may provide candidate markers for the selection of disease resistant individuals of channel catfish.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LG performed polymorphic identification and statistical analysis, and drafted the manuscript. XD performed polymorphic identification. HS, XG, YL, XB and WL participated in the coordination of the study. CH conceived the study, participated in its design and coordination.

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