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CDS Cloning, Sequencing and Bioinformatic Analysis of Vasoactive Intestinal Peptide in Wahui Pigeon

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ABSTRACT

Vasoactive intestinal peptide plays double roles as gastrointestinal hormone and neuropeptide. Due to few reports on vasoactive intestinal peptide in pigeon, the present study was aim to further explore the correlation between its molecular bioinformatics characteristics and biological function. After the desired RNA was extracted from the intestine of Wahui pigeons, the complete CDS of vasoactive intestinal peptide was obtained by PCR amplification and sequencing, in addition, a variety of bioinformatics analysis was performed to evaluate its structural characteristics. CDS of vasoactive intestinal peptide, encoding 132 amino acids, is 399 bp long in Wahui pigeon. Vasoactive intestinal peptide probably belongs to a hydrophilic peptide without any signal peptide and transmembrane structure, and mainly plays its biological function due to occupying 89.6% in cytoplasm. Furthermore, it consists of one N-glycosylation site and 17 phosphorylation sites, and the secondary structure contains 50.76% of alpha helix, 5.30% of beta turn, 9.09% of extended strand, and 34.85% of random coil. Phylogenetic tree analysis shows that Wahui pigeon hold the close relative characteristics with chicken, turkey, and *Taeniopygia guttata*. Besides its conservative evolutional characteristic, the simple structure, subcellular localization, hydrophobicity and hydrophilicity indirectly account for its extensive distribution and various functions as expected.

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Authors' Contribution
FW and YL conceived and conducted the study. FW and XT provided the methodology and resources. XH, SY, SZ, ZZ and XY made the investigation and analyzed the data. YL and FW wrote and revised the manuscript.

Key words
Wahui pigeon, Bioinformatic analysis, Cloning, CDS, Vasoactive intestinal peptide

INTRODUCTION

Vasoactive intestinal peptide (VIP) was initially isolated and characterized from porcine duodenum in 1970 (Said and Mutt, 1970). *In vivo*, VIP widely distributes in various tissues as gastrointestinal hormone and neuropeptide. VIP is considered as the paradigm of an endogenous neuroendocrine-immune mediator with therapeutic potential for a variety of inflammatory disorders such as inflammatory bowel disease (Abad *et al*., 2012; Jönsson *et al*., 2012; Wu *et al*., 2015; Iwasaki *et al*., 2019). VIP can inhibit the neurodegeneration due to loss of neurons, which may be mediated by glial cells through the production of neurotrophic factors and the inhibition of proinflammatory mediators (Deng and Jin, 2017). In addition, VIP acts as an antimicrobial peptide in the pathological process of rheumatoid arthritis, reduces the expression of pattern recognition receptor and its inflammatory signal, down-regulates the production of proinflammatory cytokines and chemokines, and counterbalances Th subsets decreasing pathogenic Th17 cells and their capacity to shift to Th1 profile (Villanueva-Romero *et al*., 2018). In the recent study, the signal pathway between nervous system and immune system mediated by VIP opens up a new way for the treatment of allergy and plays an important role in the prevention of allergic diseases (Verma *et al*., 2017). Another study demonstrates an increased transepithelial passage of live commensal and pathogenic bacteria in the colon of irritable bowel syndrome subjects, the up-regulation of VIP is important in the regulation of this bacterial translocation through the mucosa (Bednarska *et al*., 2017). So far, VIP has continually been investigated as a known neuropeptide on its aspects, the structural characteristics and biological function of VIP have been abundantly acquired as well.

Pigeon is a kind of common poultry with tender meat, high protein, and low fat in China. In recent years, the market demand for pigeons has gradually increased. However, some bad managements, for example, wet

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litter, bad ventilation, high density, improper coccidiostat consumption and contaminated feeders, exaggerate intestinal parasite problems (Ruff, 1993). The parasitism often brings about severe effects on birds including malnutrition, retarded growth, low egg production, susceptibility to other infections, and death in young birds (Radfar *et al*., 2012), these similar intestinal diseases account for a large part of pigeon's disease, so as to seriously threaten pigeon's normal breeding. As an effective anti-inflammatory factor, VIP has the effect of inhibiting T cell proliferation (Gonzalez-Rey *et al*., 2007). In addition, VIP inhibits the production of inflammatory cytokines and chemokines by macrophages, microglia and dendritic cells via its receptors VPAC1 and VPAC2 (Gonzalez-Rey *et al*., 2007). VIP is also a signaling molecule between the nervous and immune system, and plays a role in immunity, especially in local mucosal immunity (Delgado and Ganea, 2013). However, there have been few reports on molecular bioinformatics of VIP such as the related functional structures. Here, Wahui pigeons' intestines were used to obtain the coding region sequences of VIP by cloning and sequencing, and its sequence was analyzed and functionally predicted by bioinformatics software. The present results provide more detailed information for further exploration on the biological function of VIP in pigeon.

MATERIALS AND METHODS

Experimental animals

Ten healthy Wahui pigeons with half male and half female (26 days old, weighing 500±5 g) were purchased from Xixia Pigeon Farm (Nanchang, China) and provided with pigeon-starter feed and water. Raised in Animal Anatomy Laboratory for one day of fasting, pigeons were dissected after anesthetization on the next day. The intestines were quickly collected to put in liquid nitrogen, and then stored in refrigerator at -80°C. All of the procedures were performed in accordance with the Ethics Committee and Guidelines of Animal Experiments of our institute.

RNA extraction and cDNA synthesis

RNA was extracted from ileum according to Trizol method. Briefly, the frozen specimens were powdered in liquid nitrogen and homogenized in RNAiso Plus (TAKARA Bio Inc., Japan), from which 500 µl was transferred to EP tube. 200 µl chloroform was added to each tube, violently shaken for 15 s and centrifuged for 15 min (4 °C, 12000 r/min). The sample was divided into three layers: the yellow organic phase, the middle layer and the upper colorless water phase. The upper water phase was transferred to a new 1.5 ml EP tube, and then 500 µl isopropanol was added in this tube and mixed gently. The mixture was kept at room temperature for 10 min, was centrifuged for 10 min (4 °C, 12000 r/min). After the supernatant was discarded, total RNA was washed with 1 ml 95% ethanol prepared by RNase-Free ddH_2O. Then 30 µl DEPC water (TAKARA Bio Corp., Japan) was added to fully dissolve and precipitate in refrigerator at -80 °C (Thermo Scientific, USA). RNA concentration and OD value were detected by ultraviolet spectrophotometer (Beckman Corp., USA). The first strand cDNA was synthesized using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Transgen, China), and then placed in refrigerator at -20 °C.

PCR amplification and sequencing

PCR amplification primers were designed by Primer Premier 5.0 software according to VIP sequence of pigeon from GeneBank (Accession number: XM_021294865.1). CDS region of VIP was amplified by PCR using the above cDNA as a template. The PCR amplification mixture of 25 µl was as follows: 12.5 µl of 2×EasyPfu PCR SuperMix, 1 µl of each of the upstream and downstream primers (Table I), 2 µl of template, 8.5 µl of ddH_2O. PCR reaction conditions was applied as follows: pre-denaturation at 95 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 50.7 °C for 30 s, extension at 72 °C for 40 s, 35 cycles, extension at 72 °C for 8 min. The electrophoresis of PCR products was performed by using 1% agarose gel, and then the PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequence detection.

Bioinformatic analysis

The biological information of VIP was obtained by a series of predictive analysis based on CDS above. These items and software are shown as Table II.

RESULTS

CDS region of VIP

After PCR amplification, the target CDS region are 511 bp long as agarose gel electrophoresis (Fig. 1), subsequently, the nucleotide sequence was obtained by sequencing, and the open reading frame is of 399 bp length according to the sequence analysis by NCBI online.

Physicochemical properties and hydrophobicity analysis on VIP

The physicochemical properties of VIP were predicted by ProtParam software. The peptide is encoded by 132 amino acids (Fig. 2), the amino acid composition is shown in Table III, and the content of leucine is the highest with 12.9%. The molecular formula and mass of VIP are

Table I. The PCR primer sequences and predicted product length.

Item	Primer sequences (5'→3')	Product length	Tm	Purpose
VIP-CDS	TTGCTGTTTGGATGTT CTTTGAAATACTATGTGGAA	511	50.7	PCR amplification

Table II. The software or tools applied in bioinformatic analysis.

Predicted subject	Software	Website
Physicochemical properties of protein	ProtParam	https://web.expasy.org/protparam/
Hydrophobic analysis	Protscale	https://web.expasy.org/protscale/
Signal peptide analysis	SignalP	http://www.cbs.dtu.dk/services/SignalP/
Transmembrane analysis	TMHMM Serve v.2.0	http://www.cbs.dtu.dk/services/TMHMM/
Subcellular localization	Psort II	http://www.psort.org/psorb
Phosphorylation site	NetPhos 3.1 Server	http://www.cbs.dtu.dk/services/Net Phos/
Glycosylation site	NetNGlyc 1.0	http://www.cbs.dtu.dk/services/Net NGlyc/
Secondary structure	SOPMA	https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automal. pl? Page=npsa_sopma.html
Tertiary structure	Phyre2	http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi? id=index
Functional structure	SAMRT	http://smart.embl.de/smart/job_status.pl?-Jobid=116547588181601533707646MhEAszEcjM

$C_{654}H_{1032}N_{182}O_{213}S_3$ and 14948.66 u, respectively. The theoretical isoelectric point and the instability coefficient are 5.03 and 69.90, respectively. The fat coefficient and the average hydrophilicity are 78.33 and -0.703, respectively, therefore, it is probably unstable and hydrophilic peptide. According to the hydrophobicity prediction by Protscale program, it can be seen that Valine in the 67th position has the strongest hydrophobicity due to the hydrophobic value with 2.933, and Lysine in the 77th position has the strongest hydrophilicity due to hydrophilic value with -3.967. The hydrophilicity contributes to approximately 70% with larger hydrophilic region; accordingly, it should be a soluble peptide.

Table III. The amino acid composition of VIP in Wahui pigeon.

Amino acids	Number	Frequency (%)	Amino acids	Number	Frequency (%)
Arg (R)	8	6.1	Phe (F)	6	4.5
Asn (N)	7	5.3	Pro (P)	7	5.3
Asp (D)	13	9.8	Ser (S)	16	12.1
Gln (Q)	4	3.0	Thr (T)	3	2.3
Glu (E)	10	7.6	Tyr (Y)	4	3.0
Gly (G)	5	3.8	Val (V)	6	4.5
His (H)	3	2.3	Ala (A)	8	6.1
Ile (I)	3	2.3	Cys (C)	0	0
Leu (L)	17	12.9	Trp (W)	0	0
Lys (K)	9	6.8	Pyl (O)	0	0
Met (M)	3	2.3	Sec (U)	0	0

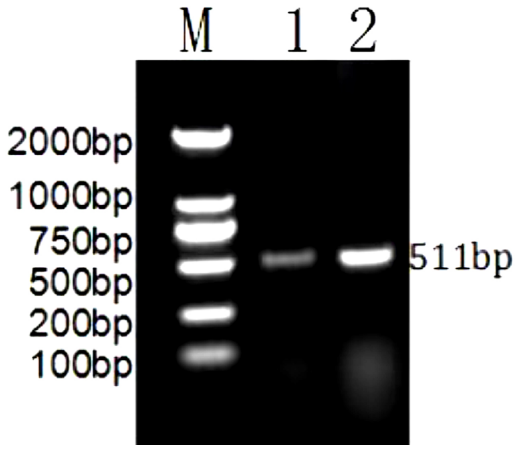

Fig. 1. The electrophoresis for PCR product of VIP-CDS in Wahui pigeon. M: 2 kb ladder marker; 1, 2: CDS product.

atg	gcc	gcc	aaa	gtt	ttg	gga	ttg	gga	aac	aga	atg	cca	ttt	gat	gga	gcc
M	A	A	K	V	L	G	L	G	N	R	M	P	F	D	G	A
agt	gaa	cct	gac	cat	gcc	cac	ggg	tca	tta	aag	tct	gaa	tca	gac	att	ttg
S	E	P	D	H	A	H	G	S	L	K	S	E	S	D	I	L
cag	aac	aca	cta	cct	gaa	aat	gag	aaa	ttc	tat	ttt	gat	ctg	tcc	aga	att
Q	N	T	L	P	E	N	E	K	F	Y	F	D	L	S	R	I
att	gat	agc	tcc	cag	gac	agt	cct	gtc	aaa	cgc	cac	tct	gac	gct	gtc	ttc
I	D	S	S	Q	D	S	P	V	K	R	H	S	D	A	V	F
act	gac	aac	tac	agc	cgc	ttt	cga	aag	caa	atg	gct	gtg	aag	aag	tac	tta
T	D	N	Y	S	R	F	R	K	Q	M	A	V	K	K	Y	L
aat	tca	gtt	tta	act	gga	aaa	aga	agc	cag	gaa	gag	ctc	aac	cct	gct	aaa
N	S	V	L	T	G	K	R	S	Q	E	E	L	N	P	A	K
ctt	cga	gat	gaa	gca	gaa	ctt	ctt	gaa	cct	tcc	ttt	tca	gaa	aac	tat	gat
L	R	D	E	A	E	L	L	E	P	S	F	S	E	N	Y	D
tct	gta	gat	gat	ctg	ctg	agc	cgc	ctc	cca	ctg	gac	ctc	tga			
S	V	D	D	L	L	S	R	L	P	L	D	L	*			

Fig. 2. The predicted amino acid sequence encoded by VIP-CDS in Wahui pigeon. The first M and asterisk represent start codon and termination codon in VIP-CDS, respectively.

The signal peptide, transmembrane structure and subcellular localization of VIP

The signal peptide of VIP was predicted using SignalP5.0 software, but no signal peptide was found in VIP of Wahui pigeon, it infers that VIP is not a secreted peptide. Meanwhile, the peptide does not have a transmembrane structure based on the prediction of TMHMM software (Fig. 3).

According to the subcellular localization by PSORT II prediction, it distributes in the cytoplasm (89.6%), cytoplasmic membrane (5.1%), periplasm (2.6%), extracellular (2.6%), and outer membrane (0.1%), respectively, therefore, it can be inferred that VIP mainly exert its function in the cytoplasm.

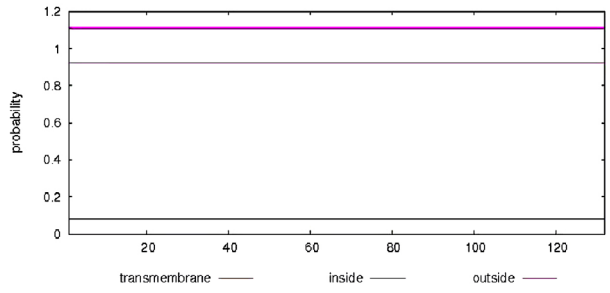

Fig. 3. Transmembrane analysis on VIP in Wahui pigeon.

Other structure prediction of VIP

As to the N-glycosylation site prediction of VIP by using NetNGlyc1.0, it was found that VIP has a potential N-glycosylation site at the 71st amino acid with a score of 0.7126 (Fig. 4). In the phosphorylation site prediction by using the NetPhos 3.1 server software online, when the threshold of potential phosphorylation site is set at 0.5, there are 17 potential phosphorylation sites in VIP, which consists of 12 Serine, 2 Threonine, and 3 Tyrosine sites (Fig. 5). There are probable six conserved protein kinase binding sites of unsp (including PKC, PKA, CKII, GSK3, and INSR in VIP), and its score has the highest value of 0.991 at 120.

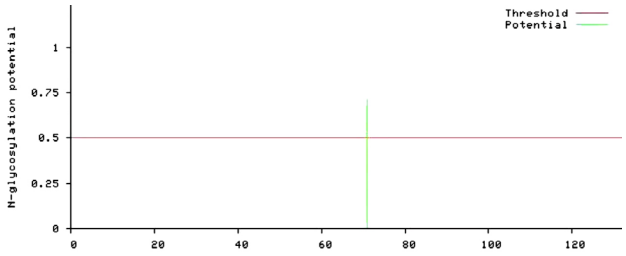

Fig. 4. N-glycosylation site analysis on VIP in Wahui pigeon.

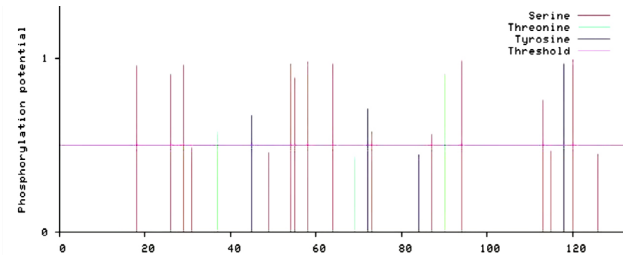

Fig. 5. The predicted phosphorylation sites of VIP in Wahui pigeon.

The secondary structure of VIP in Wahui pigeon was predicted by ExPAsY's SOPMA program. The Alpha helix of VIP has 67 amino acids, taking up 50.76%, and the Beta turn has 7 amino acids, taking up 5.30%. The extended strand has 12 amino acids, taking up 9.09%, and the Random coil has 46 amino acids, taking up 34.85%. The tertiary structural model was obtained by homology modeling of VIP with Phyre 2. The peptide is folded, bent and forms into a three-stage structure (Fig. 6).

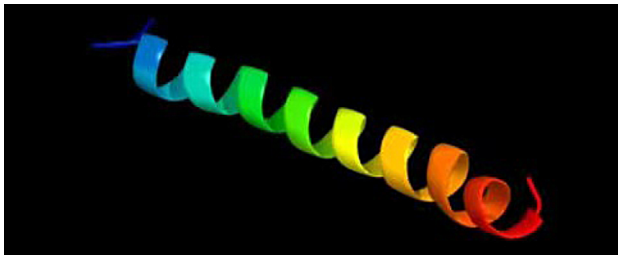

Fig. 6. The predicted tertiary structure of VIP in Wahui pigeon.

The phylogenetic evolutionary analysis of VIP

VIP sequence of Wahui pigeon was compared with other nine different animals by using MUSCLE software including *Gallus gallus* (GeneID: NM_001177309.1), *Meleagris gallopavo* (GeneID: XM_010707199.3), *Taeniopygia guttata* (GeneID: XM_030268028.1), *Danio rerio* (GeneID: NM_001114553.3), *Mus musculus* (GeneID: XM_006512448.1), *Rattus norvegicus* (GeneID: NM_053991.1), *Homo sapiens* (GeneID: NM_003381.4), *Bos taurus* (GeneID: NM_173970.3), and *Sus scrofa* (GeneID: NM_001195233.1). According to the genetic sequence analysis established among ten different animals, the phylogenetic tree can be divided into three large groups, from which Wahui pigeon locates in the same small group with *Meleagris gallopavo, Gallus gallus* and *T. guttata*, the phylogenetic evolutionary trend indicates that there are the closest relative relationship between Wahui pigeon and other three kinds (Fig. 7).

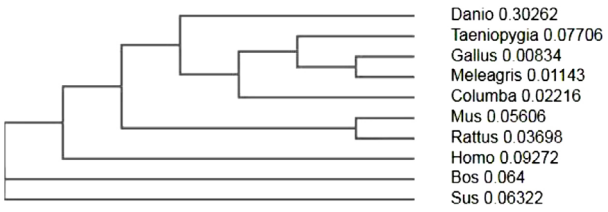

Fig. 7. The phylogenetic evolutionary analysis on VIP in Wahui pigeon.

DISCUSSION

VIP is a neuropeptide extensively distributed in tissues, it exerts pleiotropic functions in multiple systems, such as the gastrointestinal, cardiovascular, nervous, and immune systems (Villanueva-Romero *et al*., 2018; Kasacka *et al*., 2015; Jayawardena *et al*., 2017; Benitez *et al*., 2018). It has been reported that VIP can participate in regulating immune balance as an immunomodulatory and anti-inflammatory factor (Benitez *et al*., 2018; Li *et al*., 2017). Therefore, it has been considered as a potential candidate for treating various autoimmune and inflammatory diseases, and also has been effective in prevention of autoimmune diseases, such as diabetes mellitus, rheumatoid arthritis, EAE, sepsis and inflammatory bowel disease (Villanueva-Romero *et al*., 2018; Jimeno *et al*., 2010; Ibrahim *et al*., 2012; Tan *et al*., 2015; Shi *et al*., 2016; Jayawardena *et al*., 2017). In the treatment of lupus erythematosus, VIP modulates immune homeostasis of Th17/Treg by down-regulating serum levels of autoantibodies and renal levels of IL-17 and IL-6, and up-regulating renal levels of Foxp3 and IL-10, thereby improves renal functional defects, proteinuria and renal damage (Fu *et al*., 2019). VIP and its receptors are over-expressed in many common tumors, including bladder, breast, colon, liver, lung, pancreatic, prostate, thyroid, and uterine cancer, VIP and PACAP stimulate the growth of several cancer cell lines *in vitro* (Moody *et al*., 2016), as indicates VIP may affect tumor growth and differentiation. In addition, VIP widely distributes in the gastrointestinal mucosa, which has various functions such as regulating the physiology, biochemistry, immune function of the gastrointestinal tract, and resisting inflammation, taking on immunity, and relaxing muscles (Montagnese *et al*., 2015). In studies on live *Salmonella typhimurium* instead of LPS, VIP can down-regulate inflammatory mediators in human monocytes and murine macrophages (Foster *et al*., 2006; Askar *et al*., 2015; Ibrahim *et al*., 2018), thereby *S. typhimurium* probably increase its survival rate in humans and mice by VIP. According to study on the immunomodulatory effects of VIP and the increase in survival and growth of *S. typhimurium*, VIP reduced cytokines over-expressed (IL-1β, IL-6, TNF-α, IFN-γ and IL-10) in sepsis, and confirmed that VIP can be used as an adjuvant therapy for antibiotics in sepsis (Askar *et al*., 2020). The above studies demonstrate that VIP has enormous potential for the treatment of inflammatory diseases in the immunomodulation of innate immune responses.

According to the bioinformatics analyses in present study, on the one hand, Wahui pigeon is clustered with chicken, turkey, and *T. guttata*. The close relationship suggests the structure of VIP is evolutionarily conservative among these species. On the other hand, this simple structure and fine solubility account for the whole physiochemical and biological properties of VIP, and also greatly explain its extensive distribution in different tissues. It is indicated that the molecular characteristics are appropriate to play its functions in various systems.

CONCLUSIONS

In conclusion, we obtained CDS region of VIP gene with 511 bp in Wahui pigeon, and analyzed its molecular characteristics and properties, this simple molecular structure and characteristics are appropriate to take various functions in multiple systems.

ACKNOWLEDGEMENTS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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